Doctoral Thesis

Characterization of new molecular profiles in sporadic endometrial carcinoma

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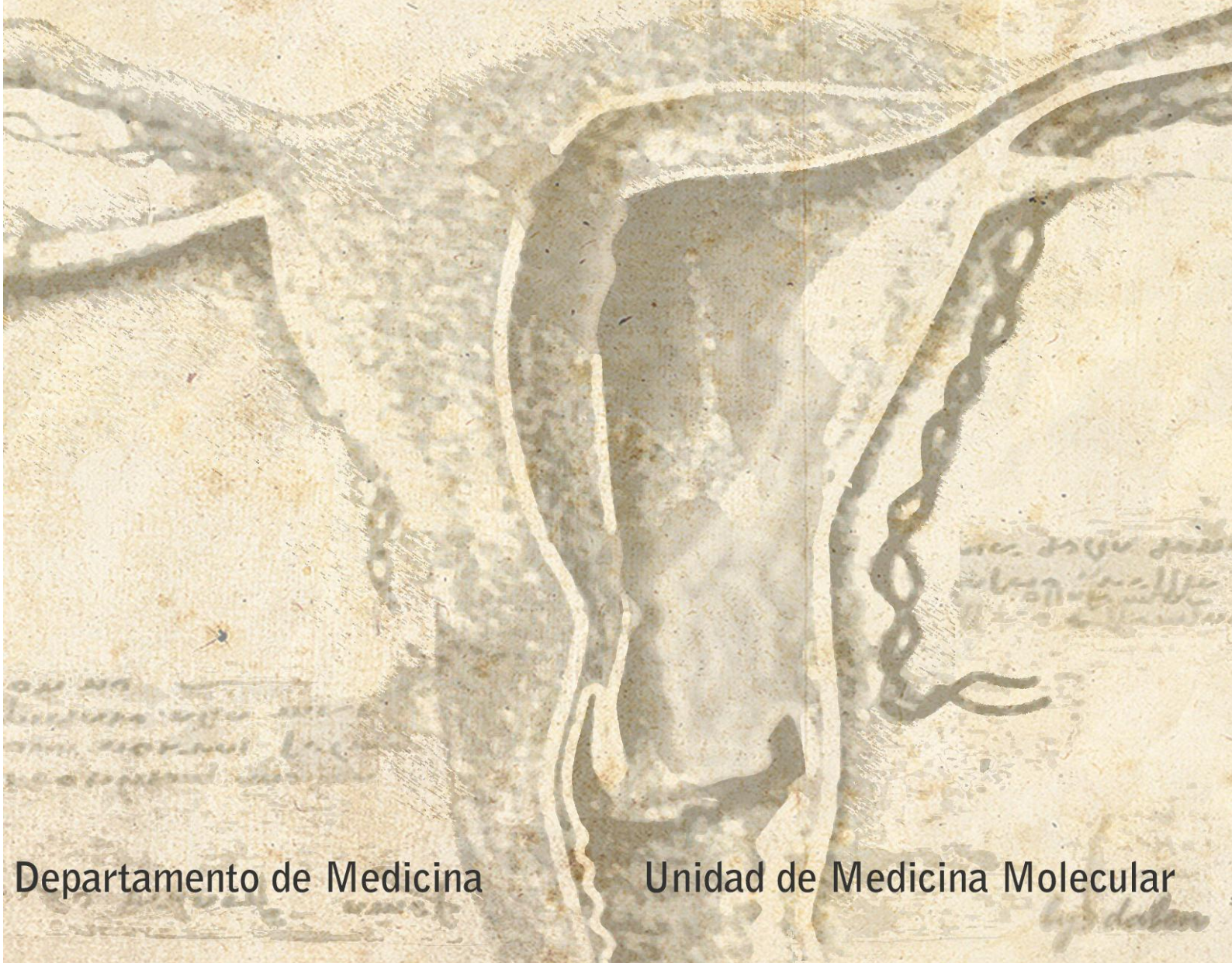

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SUMMARY

Summary

Endometrial carcinoma is the most frequent gynaecological tumour in developed countries. The origin is sporadic in 95% of them and its main risk factor is hormone exposure.

It has been proposed several classifications depending on the followed criteria to characterize sporadic endometrial carcinomas. Bearing in mind clinical-pathological characteristics, Bokhman et al., in 1983, proposed a dualistic model in which endometrial carcinomas were classified in two groups: type 1 or endometrioid carcinomas (with a good prognosis and estrogens-dependent) and type 2 or non-endometrioid carcinomas (with a poor prognosis and non-estrogens-dependent). Later, Vogelstein in 1988, developed a theory for colorectal cancer, embraced and adapted for endometrial carcinoma, which said that tumours are a consequence of an accumulation of genetic and epigenetic alterations. According to this fact, it has been observed that type 1 and type 2 endometrial carcinomas have specific molecular profiles. Type 1 carcinomas are mainly associated with MSI and mutations in *PTEN, PIK3CA, KRAS, CTNNB1*, *FGFR2* and MMR genes and type 2 carcinomas are associated with mutations in *TP53, CDKN2A,* and *CDH1* genes, loss of expression of HER2/ERBB2 and EGFR/ERBB1 proteins and loss of heterozigosity (LOH) in different chromosomes.

Currently, whole exome sequencing studies are being carried out in order to look for new target genes in different types of tumours. Two of them are *ARID1A* and *PPP2R1A* genes that are highly mutated in endometrioid and non-endometrioid carcinomas respectively. On the other hand, telomere length variations have been associated with a susceptibility of developing different kind of tumours. However, it has not been yet clarified if the telomere shortening is a cause or a consequence of tumour development and the exact role of telomerase.

Moreover, new criteria are being established with the aim of elaborate a more accurate classification that would help to better identify, characterize and treat the different types of endometrial carcinomas.

The aim of the present work has been the analysis of 14 genes implicated in tumorigenesis (including *PPP2R1A* and *ARID1A* genes); MSI; LOH; as well as the methylation of the MMR genes promoter and HDACs expression; the telomere length and *TERT-1327C>T* and *TERC-63G>A* polymorphisms analysis in a cohort of 86 sporadic endometrial carcinomas and 23 blood samples.

We have used a wide variety of techniques for the realization of our study. We have extracted DNA from tumour and blood samples, and RNA and total proteins from tumour samples. The analysis of different genes has been carried out by PCR, CSGE-Heteroduplex and Sanger sequencing using several databases in order to check if the mutations found had been previously described. For the characterization of the new mutations, we have performed RT-PCR and Western blot assays (as well as for HDAC protein expression analysis). Several prediction programs have helped us to determine the mutations pathogenicity. We also have studied LOH by qPCR and RFLP; gross alterations in MMR genes by MLPA; MMR promoter

methylation by MS-MLPA; and telomere length and telomerase polymorphisms by real time PCR. Statistical analysis has been carried out with SPSS v18.0, GenEx 5.3.6 Enterprise, and MULTBiplot programs.

We have found 213 mutations: 99 described mutations and other 114 non-previously described mutations that have been characterized in our work. The most mutated genes have been *PTEN* and *ARID1A*.

MSI has been carried out when we have obtained DNA from both tumour and blood samples of the same patient observing that it is a frequent event in our patients. In contrast with Lynch syndrome, D17S250 was the most altered marked in our population. On the other hand, we have observed that the gross alterations and the punctual mutations don´t explain the MSI and loss of expression of MMR genes.

The most methylated gene has been *hMLH1,* but methylation of MMR genes has not been consistent with either the lack of MMR proteins expression and MSI.

The HDAC2 protein has been the histone deacetylase which expression has been absent in a highest number of studied tumours. Both methylation and HDAC2 protein expression patterns have differed depending on the type of tumour.

The telomere length has not shown any relation with neither type nor grade of tumour and C and G alleles of *TERT-1327C>T* and *TERC-63G>A* polymorphisms were only slightly related to mixed and grade 3 endometrioid carcinomas respectively (the groups with shortest telomeres).

In our work, *PPP2R1A* gene has shown mutations mainly associated with serous carcinomas. We have described a new probably pathogenic mutation in the exon 2 of the gene, presented in a grade 1 endometrioid carcinoma, and a pole of polymorphisms situated in the promoter region implicated in the binding of several transcription factors. When we have analyzed *PPP2R1A* protein expression, we have observed a new pattern of protein expression at 110 kDa, maybe related to posttranslational modifications.

On the other hand, we have observed that *ARID1A* gene appears commonly mutated in endometrioid and non-endometrioid carcinomas with a high number of nosense and frameshift mutations. We have described 32 new mutations including an inframe alteration that causes a change in the mRNA splicing.

According to our results, the most adequate classification among those that have been proposed until now, is the classification that bears in mind the characteristics of the different histological subtypes. The clinical-pathological criteria are not accurate and they share a high identity with the classification by histological grades.

We have observed that grade 1 and grade 2 endometrioid carcinomas could be grouped in a low-grade endometrioid cluster showing very similar molecular characteristics. At the same time

the molecular profile of low-grade endometrioid carcinomas differs from grade 3 endometrioid carcinomas genetic profile. Grade 3 endometrioid carcinomas share characteristics with low-grade endometrioid and serous carcinomas and because of that, it is not possible to group them with any other histological subtype. Moreover, mixed carcinomas and carcinosarcomas show a molecular profile that depends on the type of their components suggesting the importance of making histological studies of this kind of tumours before their molecular analysis.

INTRODUCTION

1. THE ENDOMETRIUM: DEFINITION

The endometrium is the innermost mucous layer of the uterus, which covers it periodically depending on the estrogens and progestagens levels. It prepares the mucous membrane of the uterus to give shelter to the foetus. If the fecundation is not performed, the endometrium comes off from the uterine cavity during menstruation[1].

Endometrium is formed by a compact stratus with cilia and secretor cells, a spongy stratus with endometrial glands and a very dense basal layer consisting of conjunctive tissue (figure 1). Moreover, there are other two outer layers: myometrium, the muscular layer, and perimetrium, the serous outermost layer (figure 1). The majority of gynaecological tumours are developed in the endometrium[2].

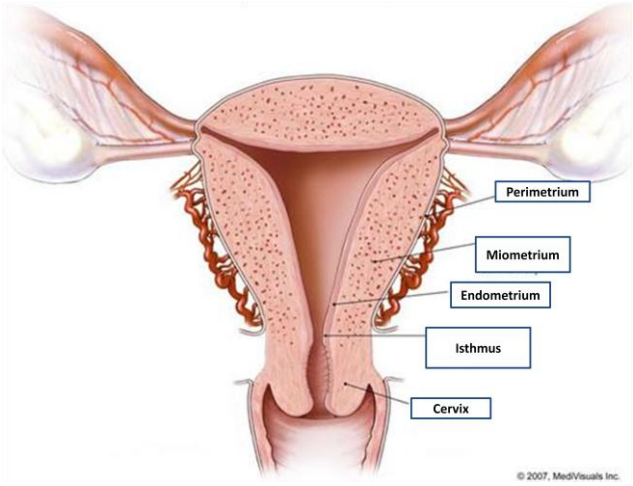

Figure 1: Schematic vertical section of a uterus in which it is observed the different layers that compose it.

2. ENDOMETRIAL CARCINOMA

2.1 EPIDEMIOLOGY

Endometrial carcinoma is the sixth most frequent tumour of the female population in the world with a 4.8% of incidence causing 74.000 deaths in 2008[3].

In developed countries, the rate of incidence increases to 5.8%, which makes gynaecological cancer the most frequent cancer among women. This increase in affected women in the past few years is caused by an increase of obesity, life expectancy, and Tamoxifen use in women[4]. The average age of diagnosis is 61 years[5,6] and 75% of the cases are detected in postmenopausal women. 95% of cases are sporadic while the other 2-5% are inherited[3].

2.2 CLASSIFICATION

2.2.1 CLINICAL PATHOLOGICAL CLASSIFICATION

Bearing in mind the clinical pathological characteristics, Bokhman proposed a dualistic model in 1983 which vastly classified sporadic endometrial carcinoma into two groups: estrogens-dependent or endometrioid and non estrogens-dependent or non-endometrioid[7].

The first group (type 1) represents 70-80% of all sporadic cases and is usually classified with low grade tumours and a good prognosis thanks to an early detection and easy treatment: the most appropriate treatment for type 1 cases is a hysterectomy[8,9]. These cases are most common in pre- and postmenopausal women and are normally preceded by a complex atypical hyperplasia or endometrial hyperplasia. This kind of tumour is characterized by the estrogens and progesterone receptors expression[10,11].

The non-endometrioid carcinomas (type 2) account for the other 20-30% of the sporadic endometrial carcinomas, which are classified by high grade and more serious prognostic tumours[12]. In non-endometrioid carcinomas there is not a hormonal receptors expression and there no previous estrogenic stimulation takes place. This type of carcinoma has a late-onset and is much more aggressive. The detection usually occurs in the later stages because endometrial polyps or endometrial atrophic do not always precede them[12,13].

If the tumour has not invaded other tissue layers, the probability of survival in next 5 years is about 96% but when it is more aggressive and there is a myometrium or lymphovascular invasion, this rate decreases to 67% and 17% respectively[14].

2.2.2 HISTOLOGICAL CLASSIFICATION

The histological classification bears in mind not only the clinical characteristics of the tumors but also takes into account the cellular type and the grade of the tumors. For that reason, it follows the criteria established by International Federation of Gynecology and Obstetrics (IFGO) and World Health Organization (WHO) in 2003 as shown below[15]:

Depending on the grade:

- Grade 1: When a maximum of 5% of the total number of cells has a solid growth neither squamous nor morular.
- Grade 2: When between the 6% and 50% of the total number of cells has a solid growth neither squamous nor morular.

- Grade 3: When more than the 50% of the total number of cells has a solid growth neither squamous nor morular.

Depending on the cell type:

- Endometrioid carcinoma:
    - Variant with squamous differentiation
    - Villoglandular variant
    - Secretory variant
    - Cilliated variant
- Mucinous carcinoma
- Serous carcinoma
- Clear cell carcinoma
- Mixed carcinoma
- Squamous cells carcinoma
- Transitional cells carcinoma
- Small cells carcinoma
- Undifferentiated carcinoma
- Sarcoma
    - Carcinosarcoma
    - Leiomyosarcoma
    - Stromal sarcoma

2.2.3 MOLECULAR CLASSIFICATION

In addition to the existence of a clinical pathological and a histological classification, there is a molecular criterion that provides a more accurate determination of different profiles, allowing for easier classification and more accurate treatment.

Endometrioid carcinomas (type 1) are most often associated with mutations in *PTEN*, *PIK3CA*[16], *KRAS*[17], *CTNNB1*[18], and *FGFR2*[19] genes as well as in genes implicated in missmatch repair mechanism (MMR) with the consequent microsatellite instability (MSI)[20]. Recently it has been noted that a high percentage of mutations exist in newly studied genes such as *ARID1A* (BAF250A)[21], *PIK3R1*[22] and *PIK3R2*[23]. In non-endometrioid carcinomas (type 2) frequent mutations also occur in *TP53*[24-26], *CDKN2A*[27], and *CDH1*[28,29] genes as well as a loss of expression of HER2/ERBB2[30] and EGFR/ERBB1[31], which is also accompanied by a loss of heterozygosity (LOH) in different chromosomes[32]. Current studies have described new mutated genes in these types of tumours such as *PPP2R1A*[33], *CHD4*, and *FBXW7*[34], especially associated to serous carcinomas.

2.3 DEVELOPMENT OF ENDOMETRIAL CARCINOMA

From a molecular point of view, the appearance of endometrial carcinoma is due to an accumulation of mutations in suppressor tumour genes, MMR genes, and oncogenes; it is also important to note the significant role that epigenetic mechanisms play. Volgestein had already elaborated this theory in 1988[35] reference to colorectal cancer. Therefore, in endometrial carcinoma, depending on the affected genes, the tumour progression will lead to different types of tumours (figure 2).

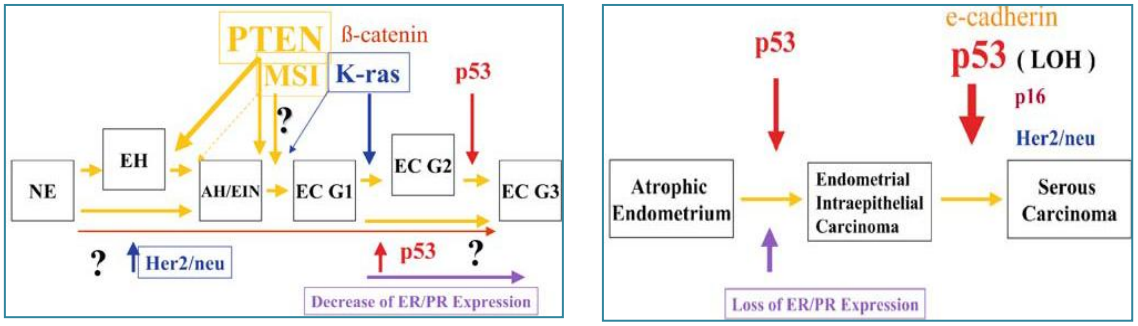

Figure 2: tumor development models depending on the molecular alterations. Left: Grade 1, 2, and 3 endometrioid carcinomas development due to the appearance of mutations in *PTEN*, microsatellite instability, mutations in *KRAS* and mutations in *HER2*, *TP53*, and *CTNNB1* as secondary events. An alternative pathway may directly lead to a high-grade tumor type by p53 mutation and her2/neu amplification, respectively. Right: Serous endometrial carcinoma development through mutations mainly in *TP53* accompanied by loss of heterozigosity, mutations in the *CDH1*, *CDKN2A*, *HER2*, and loss of expression of hormone receptors. *NE* normal endometrium, *EH* endometrial hyperplasia without atypia, *AH* atypical endometrial hyperplasia, *EIN* endometrial intraepithelial neoplasia, *EC* endometrioid carcinoma (grade 1–3)[26].

However, certain histological types of endometrial carcinoma do not have such clearly defined clinical-pathological and molecular characteristics. There are several histological types that have a strong heterogeneous molecular profile such as mixed carcinomas, grade 3 endometrioid carcinomas, and some carcinosarcomas. Due these different types, we can assume that some non-endometrioid carcinomas have originated from endometrioid tumours, which is very important to note in deep molecular analysis and the treatment process[36].

2.4 RISK FACTORS OF THE ENDOMETRIAL CARCINOMA

2.4.1 INHERITED ENDOMETRIAL CARCINOMA

The majority of endometrial carcinoma cases are sporadic and only 2% to 5% are inherited. Familiar endometrial carcinoma is mainly associated with two diseases: Lynch syndrome and Cowden syndrome. In the first case, mutations are located in MMR genes, concretely in *hMLH1, hMSH2, hMSH6* and *hPMS2*, and deletions in *EPCAM* gene[37]. Lynch syndrome is an autosomal dominant disease characterized by a high risk of colorectal and endometrial tumours development. Women affected usually manifest mutations in *hMLH1,*

hMSH2 and *hMSH6* and sometimes as a result, MSI occurs[38-43]. On the other hand, Cowden syndrome is characterized by mutations in the PTEN gene and contains harmatomas, which affect the organs[44,45]. 5% to 10% of these cases are associated with endometrial carcinoma[63,65] but the development of breast cancer is more frequent (25-50% of cases)[44,46].

2.4.2 Sporadic Endometrial Carcinoma

The primary risk of sporadic endometrial carcinoma is from the exposure of exogenous and endogenous hormones. These hormones promote cell proliferation and participate in apoptosis inhibition. The overexpression of estrogens and progesterone receptors is common in endometrioid carcinoma; in non-endometrioid carcinoma these receptors do not exist[11].

There are two other groups of potential risk factors: anatomical physiological variations-being overweight, null parity, young menarche, and late menopause. The second risk factor is diseases such as Mellitus diabetes, hypertension, polyquistic ovarian syndrome, and breast and ovarian tumours[47,48].

3. Molecular Pathogenesis of Endometrial Carcinoma

3.1 Genetics factors

3.1.1 The PI3K-AKT-mTOR Pathway

PIK3CA (phosphatidylinositol-4,5-biphosphate 3-kinase, catalytic subunit alpha) and PTEN (phosphatase and tensin homolog deleted on chromosome 10) proteins are two antagonist molecules with much interest because their interaction regulates an important pathway which results in one of the main therapeutic targets in endometrial carcinoma. These proteins maintain equilibrium in the citoplasmatic PIP3 quantity.

The *PTEN* gene is located at 10q23.3 and encodes a highly conserved protein of 403 aminoacids with a 47 kDa molecular weight. It belongs to the tyrosine kinases family and is present in five domains; the N-terminal extreme is where the phosphatase domain is located. Its main function is the dephosphorylation of the tyrosine, threonine, and serine domains of different proteins as well as the position 3 of the phosphatidylinositol 3, 4, 5-trisphosphate (PIP3), which is then converted into PIP2[49].

Furthermore, the *PIK3CA* gene is located at 3q26.32 and encodes the catalytic p110α subunit of the PI3K (phosphatidylinositol 3-kinase) heterodimer. The other subunit, p85, has the regulatory function of the complex. The PIK3CA protein consists of five domains but the catalytic domain is in charge of phosphorylating, using ATP, PIP2 forming PIP3[50,51].

PIP3 recruits proteins such PDK1 and AKT into the citoplasmatic membrane. Once there, they activate the signaling cascade with the continuous activation of different proteins. One of these proteins, mTORC1, affects some effectors, which in turn regulates several cellular processes such as cellular growth, metabolism, proliferation, survival, migration, apoptosis and angiogenesis (figure 3)[9,52].

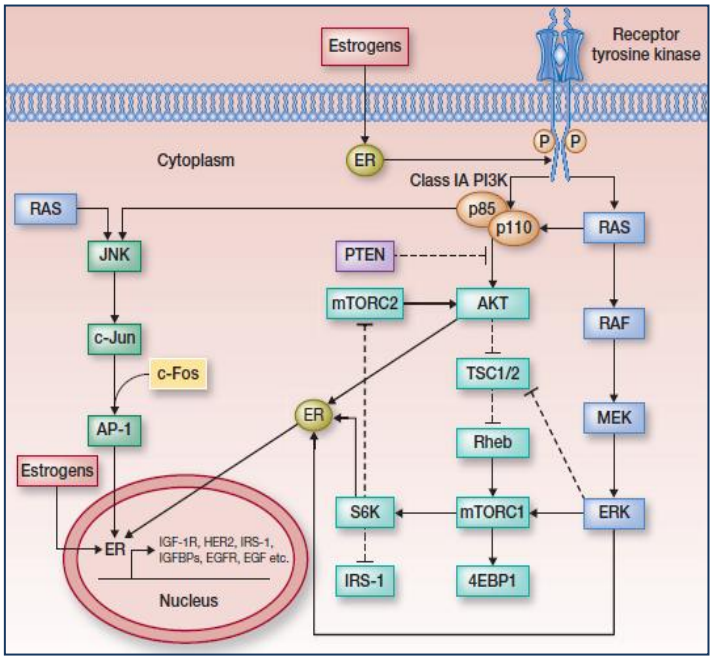

Figure 3: Scheme of the integration of the different signaling pathways highlighting that constituted by PI3K, PTEN and the AKT RAF-MEK-ERK pathway, both of them with mTORC and estrogens receptors as effectors[53].

Both *PTEN* and *PIK3CA* genes are frequently mutated in endometrial carcinoma, especially *PTEN*, which appears altered in 37-61% of endometrioid carcinomas. The missense mutations are grouped in exons which encode the fosfatase catalytic domain decreasing its function[54,55]. Moreover, the *PIK3CA* gene is usually mutated in endometrioid carcinomas (7-33%), and specially, in exons 9 and 20. Therefore, signaling is the most influenced route in endometrioid carcinoma. Non-endometrioid carcinomas can also show mutations in 5-13% of cases involving *PTEN* and 30-35% involving *PIK3CA*[16,23,54-62].

3.1.2 THE RAS-RAF-MEK-ERK PATHWAY

KRAS (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) and *BRAF* (v-raf murine sarcoma viral oncogene homolog B1) genes encode two proteins present in the MAP kinases pathway.

KRAS is a small protein of 21kDa belonging to the RAS GTPases subfamily. It is activated when there is a signal from the cell surface, such as the well-known EGFR mechanism. When this receptor is activated, the KRAS protein interacts with a GTP molecule becoming activated and then sets off the signal. Its more proximal effectors are mainly RAF kinase and PI3K[63-68] (figure 4).

BRAF is a serin-treonine kinase activated by KRAS, which is able to switch on the MAP kinases pathway by the MEK1 and MEK2 phosphorylation. Lastly, the phosphorylation of several nuclear and citoplasmatic factors cause the activation of cellular process such cellular proliferation, survival, differentiation, motility, and angiogenesis[66-69] (figure 4).

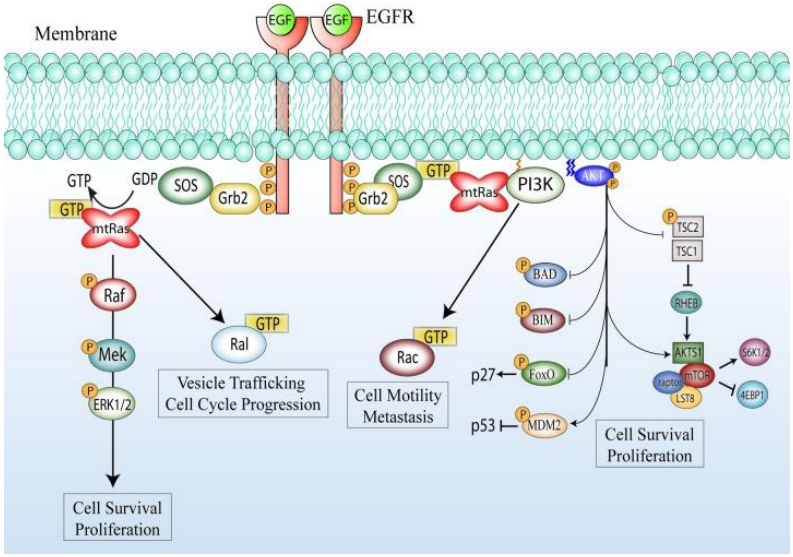

Figure 4: RAF and PIK3CA activation by the RAS protein in response of the activation of the EGFR receptor. It is also observed the activation of GTPases such as RAC and RAI responsible for the modification of the cytoskeleton and the vesicle traffic[70].

The *KRAS* gene has 6 exons but the described somatic mutations present in tumours are located in codon 12 and 13 (exon 2) and codon 61 (exon 3). The changes that occur here make GTP permanently linked to KRAS and therefore KRAS is always activated. Particularly in endometrial carcinoma *KRAS* gene is not a much altered gene. The frequency of mutation is roughly 14% but in endometrial carcinoma this number increases to 18%[71,72]. On the other hand, BRAF is encoded by 18 exons with the 11th and 15th exons being hotspots. The mutations located in these hotspots maintain the kinase activity and therefore, the pathway constantly activated deregulating every cellular process as a consequence. These hotspots are not very common in endometrial carcinoma (1-4% independently of the type)[73].

3.1.3 THE WNT PATHWAY

The WNT pathway regulates processes such as proliferation, differentiation, and cellular polarity. The best-known mechanism is that which implicates the Frizzled (Fz) receptors and β-catenine[74].

β-catenine is a protein regulated by a complex that binds it and promotes its degradation by proteosome. When Wnt interacts with the Fz receptors, Dishevelled (Dsh) proteins prevent its degradation and therefore β-catenine accumulates at cytoplasm[74]. Then, it is translocated to the nucleus where it coactivates several transcription factors that enhance the transcription of the Wnt response genes[75]. This pathway is mainly altered by mutations in *CTNNB1*, the gene that encodes β-catenine, chiefly in endometrioid carcinoma (45%). This gene, located at 3p22.1, is formed by 15 exons but exon 3 is where most of the mutations have been observed which impedes the degradation of β-catenine by proteosome. This protein has a molecular weight of 88 KDa and plays an important role in the maintenance of the cytoskeleton when interacting with E-cadherine[74].

E-cadherine is encoded by *CDH1* gene, situated at 16q22.1. It has 16 exons and the resulting protein has a molecular weight of 120 KDa. There are three main domains: an extracellular domain that interacts with Ca^{2+}, one transmembrane domain and a citoplasmatic domain. This last one interacts with α-actin cytoskeleton through catenines including β-catenine. The complex results are essential for the cellular adhesion and motility providing a homeostasis status in the tissue (figure 5)[76-78].

CDH1 mutations are not very frequent in somatic endometrial carcinoma but are related to motility cell changes facilitating in invasion, metastasis, and the progression of the tumor leading to a worse disease prognosis. The most common event is the LOH (22-57%) and promoter hypermethylation (21-40%)[79].

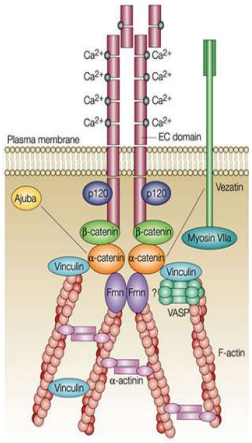

Figure 5: Complex formed by the interaction between the transmembrane protein E-cadherin, in which it is observed the extracellular domains (EC) whose Ca^{2+} binding allows its homodimerization, and the β-catenine that mediates the binding to the α-catenine and allows the recruitment of a wide quantity of proteins of the cytoskeleton[80].

3.1.4 Other Genes Implicated In The Endometrial Carcinoma Pathogenesis

3.1.4.1 The *TP53* Gene

TP53 is a suppressor gene located at 17p13 and formed by 11 exons[81]. The first one is not coding. The P53 protein has 393 amino acids divided in three domains with five conserved regions. One of the main domains is the transactivation, which interacts with transcriptional factors, acetiltransferases, and MDM2[82,83]. This protein quantity is regulated by the MDM2 protein and postraductional modifications, which interact with P53 through negative feedback and then facilitates ubiquitination and degradation by proteosome[83,84].

When there is a disadvantaged cellular situation other proteins modify P53 through postraductional changes, which avoid interaction with MDM2 and therefore its degradation facilitates in the P53 accumulation. P53 then makes a homotetramer, which binds to DNA activating gene transcription (figure 6)[83].

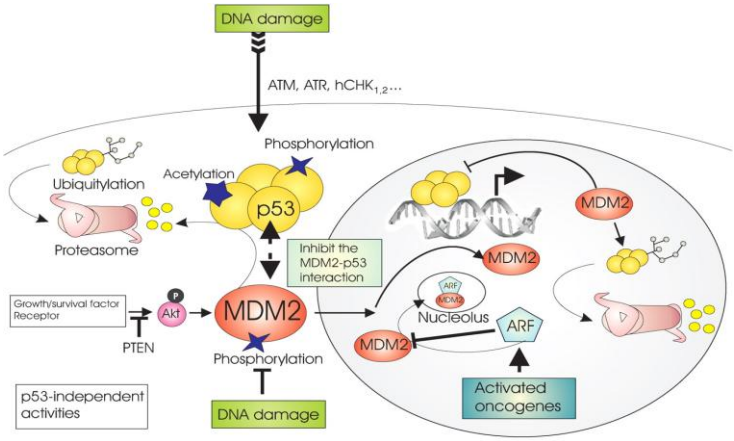

Figure 6: Regulation of the p53 cellular levels by interaction with MDM2 and the posttranslational modifications. Under normal conditions, the cellular p53 levels are regulated by its interaction with MDM2 and vice versa. Under a stressing situation that can cause DNA damage, p53 is post-translationally modified in order to prevent its degradation by proteosome, unlike MDM2, which is phosphorylated and marked for degradation with the aim of p53 goes to the nucleus and activates the transcription of different genes required for the cell response to this unfavorable situation[85].

P53 is implicated in several important cellular mechanisms such cellular cycle control[86-90], apoptosis induction[91-94], genomic stability maintenance[95,96], and angiogenesis inhibition[97]. The *TP53* gene is frequently mutated in serous endometrial carcinoma (53-90%)[24,25,98-100] and the mutations tend to appear in the early stages of tumorigenesis. In endometrioid, carcinoma is altered in a smaller percentage of tumours (12-23%)[24,99,101] being higher in endometrioid grade 3. These mutations correlate with MSI[26] but not with mutations in *PTEN*, *KRAS*, and *CTNNB1* genes[24].

3.1.4.2 THE *CDKN2A* GENE

The *CDKN2A* gene (cyclin-dependent kinase inhibitor 2A) is located at chromosome 9p21 and codifies the 16 kDa phospholipoprotein p16[102].

P16 is a tumoral suppressor that is part of the kinase cycline dependents inhibitor protein (INK4A). It binds CDK4 and CDK6 kinase cyclines and inhibits the CDK-cycline D complex catalytic activity, a G1 phase checkpoint regulator. The absence of p16 is basis of the binding, phosphorylation, and inhibition of Rb protein by kinases, which then changes the G1 to S phase transition resulting in cellular proliferation (figure 7)[103-105].

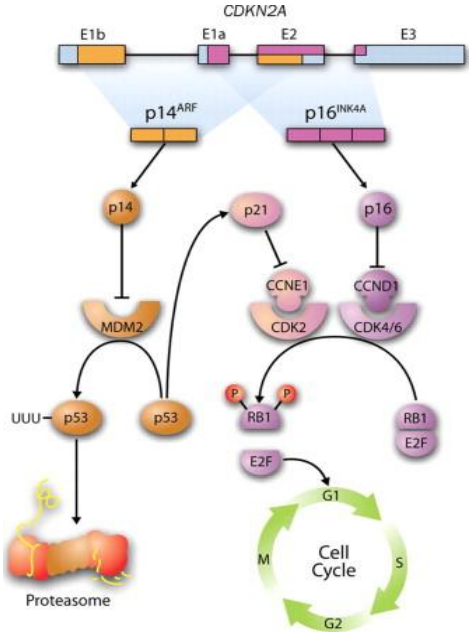

Figure 7: Complex formed by the interaction between the transmembrane protein E-cadherin, in which it can be seen the extracellular domains (EC) whose binding to Ca^{2+} allows homodimerization, and the β-catenine that mediates binding with the α-catenine allowing the recruitment of a large amount of citoskeletal proteins[80].

This deregulation occurs due to genetics and epigenetics processes such homozygous deletions, punctual mutations, and methylation of *CDKN2A* promoter[106-108], which is cause for the loss of p16 expression very common in non-endometrioid carcinomas (20-70%). These types of alterations are associated with more aggressive tumours with a poorer prognosis[32,109-116].

3.1.4.3 THE *PPP2R1A* GENE

The *PPP2R1A* gene codifies the α isoform of the protein phosphatase 2A subunit A (PP2A), one of the four main serine threonine phosphatases[117,118].

The protein PPP2R1A, with 65 kDa and 590 aminoacids, resides in the scaffolding subunit of the complex and is in contact with subunit B (regulator subunit) and subunit C (catalytic subunit) (figure 8). PP2A complex interacts with a wide variety of effectors such as c-MYC, BCL2, ERBB2, AKT, MEK, WNT, p53 and RAF. It therefore participates in different pathways implicated in cell cycle, mitosis, DNA reparation as a response against hypoxia and even in neoplastic transformation associated to virus[119-124].

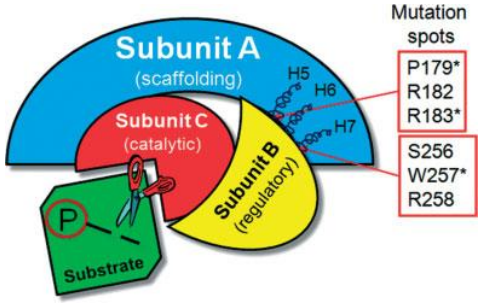

Figure 8: PP2A complex representation in which it can be observed the A, B, and C subunits and some of the most frequent pathogenic described mutations located in the HEATS 5, 6, and 7 situated in the region of interaction between the subunits A and B[125].

In general the protein has 15 HEAT (Huntington-Elongation-A subunit-TOR) tandem repeats which gives it its helicoidally shape. HEATS from 2 to 7 are in contact with subunit B and two hotpots exist in H5, H6 and H7, codified by exons 5 and 6[125]. All of mutations indentified are heterozigous missense changes that can alter the interaction between subunit A and B and the complex destabilization. However, the mutations percentage is not very high but is mainly concentrated in non-endometrioid carcinomas, particularly in serous carcinoma (43.2%) and carcinosarcomas (21.4%). Mutations have been found in other types of tumours but with a low frequency[36,126,127].

3.1.4.4 THE *EGFR* GENE

EGFR (epidermal growth factor receptor) is a tyrosine kinase expressed in the cell membrane surface of the normal cells[128]. It is formed by three domains: the extracellular, that is responsible for the ligand binding (mainly EGFR), and the intracellular, which has the tyrosine kinase activity upon some effectors such PLC-γ, MAPK and GAP. This process is activated when the ligand binds to EGFR and EGFR phosphorylates effectors, starting the pathway accountable for different cellular responses[129-131].

EGFR is frequently overexpressed especially in serous uterus carcinoma (36-56%)[31,61]. This signifies a bad prognosis and it is thought to be due to mutations in exons 18-21 which encode the tyrosine kinase domain[132-134]. However a high level of mutations in this kind of cancer have not been found therefore it is most likely caused by other molecular mechanisms[61].

3.2 DNA REPARATION

3.2.1 MISMATCH REPAIR MECHANISM (MMR)

Mismatch repair mechanism is a highly conserved regulation process responsible for the correction of the base-base and extra-helicoidal loops resulting from small insertions and deletions caused during DNA replication.

In eukaryotic organisms, MMR system is formed by two complexes: MutS and MutL. MutSα is composed of hMSH2 and hMSH6 and recognizes base changes as well as loops resulting from a base insertion or deletion (IDLs). MutSβ (hMSH2 and hMSH3) can also recognize IDLs but with more affinity for those which are more complex such 2 to 4 bases insertions. MutL heterodimers are recruited by MutS complexes and are guided to the wrong area. They are formed by hMLH1 dimerized with hPMS2 (MutLα), which is able to take part in the big and small IDLs, hPMS1 (MutLβ) or hMLH3 (MutLγ) with an unidentified role in the MMR (figure 9)[135].

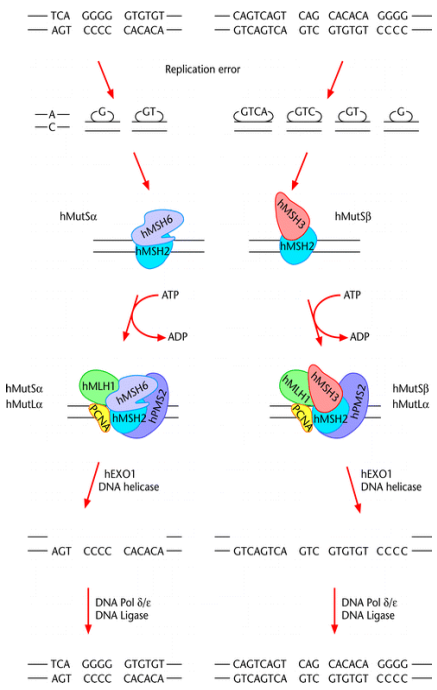

Figure 9: Mismatch repair mechanisms in which are involved the MutSα-MutLα and MutSβ-MutLα complex and the three main steps of the correction process: error recognition, complex assembly and degradation of the wrong strand and synthesis of the new one[136].

Mutations in the MMR are related to colorectal, endometrial, and ovarian cancer similar to Lynch syndrome. The most mutated genes in colorectal cancer are *hMLH1* (60%) and *hMSH2* (35%)[137], which is contrary to endometrial carcinoma associated with Lynch syndrome in which the risk of development is 73%, 29% and 31% when mutations are present in *hMSH6*, *hMSH2* and *hMLH1* genes[138].

3.2.2 MICROSATELLITE INSTABILITY (MSI)

Microsatellites are short and repetitive sequences highly distributed in the genome and more frequently in junk DNA areas. 50.000 and 100.000 of these sequences have been found in non-coding regions and only a smaller quantity in coding regions.

In general, these sequences are very polymorphic and are representative of stable and inheritable differences inside the population[139].

Microsatellites represent a high susceptibility of acquiring spontaneous insertion and deletion mutations of a repetitive unit. This event is known as Microsatellite Instability (MSI)[139].

MSI occurs due to mistakes in DNA replication because of polymerase slicing which causes expansions and contractions of the sequences. Under normal conditions, the MMR solves these mistakes, but when a problem exists in the mechanism it does not function properly and these alterations remain unfixed. These alterations can lead to cancer when the affected regions are located in genes implicated in vital cellular functions[140].

The MSI phenotypic determination depends on the number of altered markers while comparing DNA from two different cellular populations (for instance blood and tumor tissue):

- High grade MSI (MSI-H): when 30% or more of studied markers show MSI.
- Low grade MSI (MSI-L): when less than 30% of studied markers show MSI.
- Microsatellite stability (MSS): when all markers are stable[141].

MSI is presented in 75% of endometrial carcinoma associated to Lynch syndrome[142] and 20% when it is sporadic[38,143,144]. However, the detection of punctual mutations when repairing genes is lower than the expected due to the presence of MSI. This fact suggests the existence of other mechanisms inhibiting the MMR system. For example, in endometriod carcinomas a high level of *hMLH1* hypermethylation has been detected which could act as a second hit[145-149]. A small portion of endometrioid carcinomas with MSI

shows mutations in *hMSH6* and *hMSH2* including in *hMSH3*, but this could be a consequence of the MSI and not a cause[150-153].

3.3 EPIGENETICS FACTORS

3.3.1 PROMOTER METHYLATION

Methylation is a post-transcriptional covalent modification consisting of the attrition of a methyl group from S-adenosilmetionine (SAM) to 5´ site in a Cytosine adjacent to a Guanine (CpG). As a result, 5-methyl-2´-deoxycytidine is formed (figure 10). The charged enzymes are DNA methyltransferases (DNMTs) (figure 10)[154].

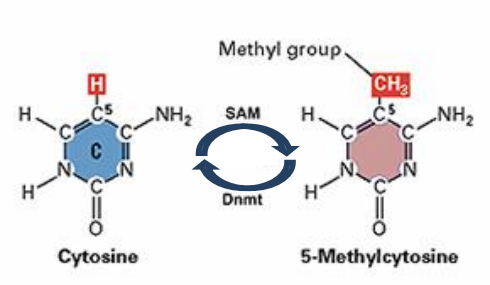

Figure 10: Methylation process from the S-adenosylmethionine (SAM) to the 5´position of a Citosyne nucleotide by the DNA methyltransferases enzymes (DNMTs) obtaining a 5´-methylcytosine as a result.

Generally, methylation affects rich CG nucleotides zones located at the 5´-UTR region and the first exons of genes, these are called CpG islands. The modification represses the DNA transcription in two ways: on one hand it inhibits the binding of transcription regulating molecules to CpG islands and on the other hand the methylated regions are recognized by transcription repressing proteins which bind them[155].

In normal conditions, methylation inhibits some gene transcription, repetitive sequences, and heterochromatin. In tumours, this methylation takes place mainly in suppressor genes, MMR genes, cellular cycle controlling genes, genes implicated in apoptosis, etc. Hypomethylation can also be problematic when dealing with oncogenes and transposons.

In endometrial carcinoma hypermethylation has been observed mainly in genes implicated in MMR, for instance in *hMLH1*[79,145,146] as a second hit. It is also frequent in the *PTEN* gene in 20% of endometrial carcinoma cases[156] and acts as an additional mechanism as well as in *APC*[157], *CDH1*[158,159] and *CDKN2A*[160] genes. Sometimes its origin is not clear and it is uncertain if the epigenetic modification is a cause or a consequence of the tumourigenesis.

3.3.2 HISTONE COVALENT MODIFICATION

Histones are a group of proteins whose main role is the packing of DNA and maintaining the chromatin structure. They comprise an octameric nucleosome core particle in which 147 bp of DNA wrap around. Each octamer consists of two groups of 4 histones: H2A, H2B, H3 y H4. Between the nucleosome and nucleosome there is a linker DNA, which forms loops and binds histone H1 charged with stabilizing the structure.

Histone N-terminal tails reside in approximately 20 to 30 amino acids all with a positive charge due to the Lysines residues. These tails are subjected to post translational modifications such Lysine acetylation, Lysine and Arginine methylation, phosphorylation, ubiquitination, sumoylation, and ADP-ribosylation. All these mechanisms are reversible and can change during a short interval of time providing chromatin with a high dynamism[161-163].

Of all the post-transcriptional modifications, the best characterized is the Lysine acetylation and deacetylation which require DNA transcription, activation, and deactivation[164]. The proteins in charge of performing this event are the nuclear enzymes histone acetylases (HATs) and histones deactylases (HDACs) (figure 11)[165].

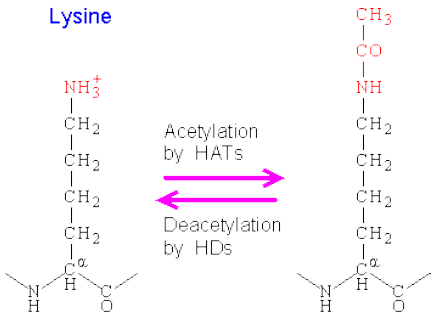

Figure 11: Processes of acetylation and deacetylation of a Lisine by the histone actylases (HATs) and the histone deacetylases (HDACs) respectively.

There are several types of HDAC with different cellular and tisular localization. HDAC1, HDAC2, and HDAC3 pertain to class 1. They are mainly situated in the nucleus but are highly ubiquous and their sequences consist between 350 and 500 amino acids[166].

In a normal endometrium, the HDAC1, HDAC2, and HDAC3 expression varies with the cell cycle[167]. Most of endometrial carcinoma shows an overexpression of three isoforms in the cell nucleus and especially in non endometrioid carcinomas: HDAC2 in 95%, HDAC3 in 83%, and HDAC1 in 61%[168]. Furthermore, there is a HDAC1 loss of expression in some endometrial carcinomas and this can lead to a more serious prognosis[167].

3.3.3 ATP-DEPENDENT CHROMATIN REMODELING

ATP dependent chromatin remodeling is performed by remodeling complexes constituted by enzymes that use the ATP energy to undo DNA-nucleosomes contacts, move nucleosomes along the DNA, and add or delete nucleosomes avoiding the basal transcriptional machinery assembly[169]. Therefore, mutations or loss of expression of these enzymes cause tumours or apoptosis process due to the cell cycle deregulation[170].

Based on the structural domains, there exists 4 families of ATPases chromatin remodeling in mammals: SWI/SNF (switching defective/sucrose non-fermenting), ISWI (imitation SWI), NuRD (nucleosome remodeling and deacetylation)/Mi-2/CDH and INO80 (inositol requiring 80). These complexes are implicated in more cellular mechanisms. For example INO80 and SWI/SNF families participate in double strand breaks (DSB) and nucleotide excision repair (NER)[171].

3.3.3.1 ARID1A

ARID1A (AT-rich interactive domain 1A) is located at 1p35.5 and codifies BAF250a, a protein involved in the SWI/SNF complex of ATP dependent chromatin remodeling.

BAF250a interacts with different proteins including BRG and BRM, which have ATPase activity and form the catalytic subunit of the complex. The BAF250 protein contains several domains, the DNA binding domain being one of the most important because it allows for the SWI/SNF complex to bind to DNA AT-rich regions. There are also two signals in the protein NLS (nuclear localization signal) and NES (nuclear export signal) important for protein function in the correct localization[172].

The SWI/SNF complex can be considered an epigenetic regulation factor because of its various roles in the gene expression, cellular growth inhibition, cell differentiation, tissue development, and tumour suppression. All of these functions are possible due to its interaction with a wide variety of effectors (figure 12)[173-175].

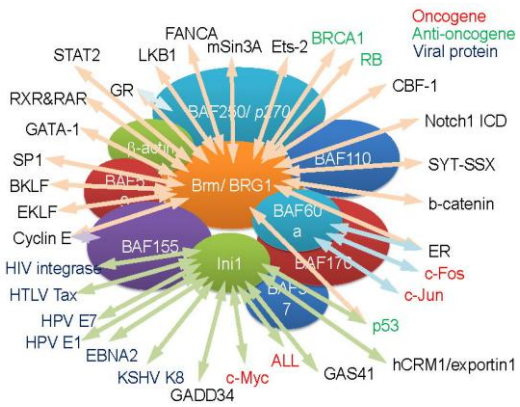

Figure 12: Scheme of the interactions that carry out some of the components of the SWI/SNF complex with a wide variety of molecules involved in different cellular processes.

It is thought that BAF250a inactivation causes an increase in cellular progression via c-myc, contributing to the uncontrolled cancerous cell proliferation.

The *ARID1A* gene is mutated in a large variety of human tumours mainly those related to the endometrium. It is not yet clear if *ARID1A* mutations are created before or after the start of tumorigenesis but it has been observed that the gene inactivation occurs in the early stages of the tumor progression in both clear cells and endometrioid carcinomas[21,176-179].

In some types of tumours, the loss of expression is much higher than the percentage of mutation and it has also been found that there exists other kinds of mechanisms, which can lead to promoter methylation and loss of heterozigosity[180,181].

3.4 THE TELOMERES

Telomeres are DNA non-coding regions located at the end of eukaryotic chromosomes. In humans, they consist of TTAGGG tandem repeats forming two mixed strands. Its 3'end is rich in Guanines and Cytosines asymmetrically distributed acquiring a secondary loop structure stabilized by specific binding protein (figure 13)[182,183].

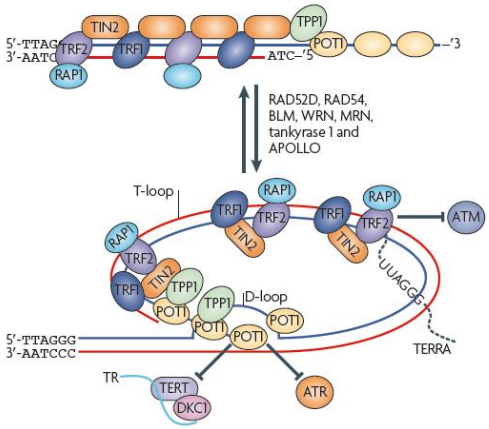

Figure 13: Linearized telomeric end (on the top) and telomeric end forming a loop (on the bottom) with their associated proteins[184].

In germ cells[185] and developing tissues[186] an active mechanism of telomere replication exists, the telomerase enzymatic complex. There is evidence that telomerase enzymatic complex is also present in endometrial cells[187], epidermis basal layer[188], and hematopoietic system[189]. In certain cells, telomerase may be activated during specific life stages[190,191].

Telomerase is formed by three main components: the reverse transcriptase TERT, the ribonucleic acid TERC, and an auxiliary protein called dyskerin (DKC1) [360,361]. In cells with active telomerase, the telomeric DNA (tDNA) remains in a state of dynamism called "telomere homeostasis"[192]. In each cycle of cell division, the telomere length decreases even though the cell has the capability to complete its replication. If this did not occur, the cell could acquire a state of immortality, which would lead to an excessive cell proliferation. In those cells in which the telomerase system is suppressed, each replication and subsequent cell division loses 100 to 200 bp at the 5' end extreme. This telomeric sequence corresponds to the gap left by the Okazaki fragment. Thus, after a certain number of divisions (between 40 and 70) the telomere length becomes critical (2.5 kb) and reaches the point of Hayflick[193]. This process leads the cell to a senescence state in which it is metabolically active and viable, but cannot proliferate[194].

Recently, it has been found that senescence has a main role in cancer development. On the contrary, telomerase is overexpressed in tumor cells, which causes cell immortalization[195]. Despite this fact, telomerase activity is not alone responsible for tumour cell converse. However, its presence is necessary. Many studies that have been challenging this theory arguing that it depends on the type of tumor; it is more contingent on the presence or absence of telomere length and telomerase activity.

However, in gynecological tumours, telomerase activity increases in the earlier stages but decreases after performing chemotherapy treatments. In ovarian cancer, telomeres are short despite the high telomerase activity. This increases the chances of developing sporadic and inherited ovarian carcinoma[196,197].

HYPOTHESIS AND AIMS

Endometrial carcinoma is the most frequent gynaecological tumor in developed countries[3]. 95% of cases are sporadic[3] and the main risk factor is hormone exposure[11].

Bokhman, in 1983 proposed a dualistic model which divided sporadic endometrial carcinoma into two main groups according to clinical-pathological characteristics: estrogen dependent (type 1 or endometrioid), which occurs between 70 to 80% of all total cases, and non-estrogen dependent (type 2 or non-endometrioid) which occurs in 20 to 30%[7] of cases. Moreover, it has been observed that both groups are associated with certain molecular characteristics. In type 1 or endometrioid carcinomas there are frequent *PTEN*, *PIK3CA*[16], *KRAS*[17], *CTNNB1*[18], *FGFR2*[19], and MMR genes mutations as well as MSI[20]. In type 2 or non-endometrioid carcinomas there are frequent *TP53*[24-26], *CDKN2A*[27], and *CDH1*[28,29] gene mutations as well as a loss of HER2/ERBB2[30] and EGFR/ERBB1[31] protein expression and LOH[32]. Exome sequencing studies have identified new genes implicated in tumorigénesis such as *ARID1A*[176] and *PPP2R1A*[127], with a high number of mutations in endometrioid and non-endometrioid carcinomas respectively.

Later, it has been proposed other classifications according to the histological characteristics of tumours. Following IFGO and WHO criteria, endometrial carcinomas can be classified according to the differentiation grade of their cells (grade 1, 2, and 3) or on the histological characteristics of each subtype[15,198,199]. This last option yields a more accurate classification in which each tumour subtype is considered as an individual group with its own molecular characteristics and specific treatment.

Epigenetic modifications also play an important role in the endometrial carcinoma development. On one hand, the *hMLH1* gene promoter hypermethylation is one of the main causes of MSI[145-149]. On the other hand, changes have been observed in the class I HDAC protein expression profile present during tumorigenesis. Concretely, the loss of HDAC1 protein expression is associated with a poor tumour prognosis and a small chance of survival for the patient[167].

Finally, telomere length has been related to the likelihood of developing different tumours as well as the stage of disease. In endometrial carcinoma, the role of telomeres and the telomerase complex has not been clarified[195-197,200].

Therefore, in our subject matter, we have analyzed the different aspects involved in sporadic endometrial carcinoma development from five main objectives:

- **First objetive:** *PTEN*, *TP53*, *CDKN2A*, *CDH1*, *PPP2R1A*, *ARID1A*, *PIK3CA*, *CTNNB1*, *KRAS*, *BRAF,* and *EGFR* genes study.
- **Second objetive:** MSI, promoter regions methylation, gross alterations, and punctual mutations in *hMLH1*, *hMSH2,* and *hMSH6* genes study.

- **Third objetive:** HDAC1, HDAC2, and HDAC3 proteins expression study.

- **Fourth objetive:** analysis of telomere length and study of its possible relation with *TERT-1327C>T* and *TERC-63G>A* polymorphisms.

- **Fifth objetive:** establishment of differences among the molecular profiles of the different sporadic endometrial carcinoma types according to their clinical-pathological characteristics (type 1 or endometrioid carcinomas and type 2 or non-endometrioid carcinomas), histological grades (grade 1, 2, and 3), and histological subtypes (endometrioid carcinomas, serous carcinomas, clear cell carcinomas, mixed carcinomas, and carcinosarcomas).

PATIENTS AND METHODS

Our research has been influenced by a very credible study conducted in 2006. Dr. Mercedes Núñez Lozano previously studied tumours 1 through 41 while we studied *PPP2R1A, ARID1A, EGFR,* and *hMSH6* genes, telomere length and *TERT-1327C>T* and *TERC-63G>A* polymorphisms studies in every sample. In our study, we have analyzed all the molecular aspects in cases 42 to 86. Moreover, we changed the SNPD3 in silico program for GVGD program and analyzed missense mutations with ESE-Finder and RESCUE-ESE programs. We used the program TFSEARCH that aided in the characterization of promoter sequences in cases where the there were changes in the 5´-UTR region applied the microRNA program for the characterization of certain mutations located in 3´-UTR.

1. PATIENTS AND SAMPLES

We obtained, prior to informed consent, 86 fresh endometrial carcinoma tumours belonging to Pathologic Anatomy Service of the Hospital Clínico Universitario de Salamanca. The average age of the patients was 69.36±10.29. Every tumour was classified by a histological subtype and grade according to the IFGO and OMS criteria (table 1):

Table 1: Number of samples analyzed of each type and grade of tumour according to the study carried out by the Pathologic Anatomy Service of the Hospital Clínico Universitario de Salamanca.

Type of tumour	Grade	Subtype of tumour	Number of tumours	Percentage (%)
Type 1 or endometrioid	Grade 1	G1 Endometrioid	31	36.05
	Grade 2	G2 Endometrioid	24	27.91
Type 2 or Non-endometrioid	Grade 3	G3 Endometrioid	8	9.30
		Serous	8	9.30
		Clear cell	1	1.16
		Mixed	2	2.33
		Carcinosarcoma	12	13.95
		TOTAL	86	100.00

We also extracted blood samples from 28 of the total patients in the Gynecological Service of the same hospital: 10 endometrioid carcinomas grade 1 (cases 3, 21, 40, 41, 42, 43, 50, 65, 68, and 79), 10 grade 2 (cases 1, 15, 17, 37, 44, 45, 54, 55, 69, and 86), 2 grade 3 (cases 52 and 80), 3 serous tumours (cases 25, 49, and 70), 1 mixed carcinoma (case 23), and 2 carcinosarcomas (cases 16 y 53).

Genomic DNA from the tumours and peripheral blood samples was extracted by standard phenol-chloroform procedures. Proteins from the tumour samples and cellular cultures were extracted by standard procedures with RIPA and API reagents in the first case and lysis buffer with proteases inhibitors in the second one. RNA extraction from tumour samples was carried out following the method defined by Chomczynski y Sacchi[201].

2. MOLECULAR STUDY OF GENES ASSOCIATED TO EC

In order to analysis the genes most often mutated in endometrial carcinoma, we performed different techniques depending on the gene and its characteristics. In several genes, not every exon was studied because the focus was placed towards exonic and intronic regions where the more important of the pathogenic mutations exist (table 2).

Table 2: Analyzed regions in each gene and method used for their study.

TYPE	GENE	CODING EXONS	ANALYZED EXONS	PCR	DIRECT SEQUENCING	CSGE-Heteroduplex
TUMOUR SUPRESSORS	***PTEN***	1-9	1-9	YES	5, 6, 7, and 8	1, 2, 3, 4, and 9
	TP53	1-10	4-10	SÍ	4-10	---
	CDKN2A	1-2	1-2	SÍ	1-2	---
	CDH1	1-16	1-16	SÍ	---	1-16
	PPP2R1A	1-15	1-15 or 5 and 6	SÍ	1-15	5 and 6
	ARID1A	1-20	1-20	SÍ	---	1-20
ONCOGENES	***PIK3CA***	1-20	7, 9, and 20	SÍ	---	7, 9, and 20
	CTNNB1	2-16	3	SÍ	3	---
	KRAS	1-6	2 and 3	SÍ	2 and 3	---
	BRAF	1-18	11 and 15	SÍ	---	11 and 15
	EGFR	1-28	18-21	SÍ	18-21	---
MMR GENES	***hMLH1***	1-19	1-19	SÍ	---	1-19
	hMSH2	1-16	1-16	SÍ	---	1-16
	hMSH6	1-10	1-10	SÍ	---	1-10

All the detected mutations in the DNA were checked from peripheral samples in order to discard a possible inherited origin.

2.1 DNA FRAGMENTS AMPLIFICATION BY PCR

The amplifications of the different studied fragments were carried out by PCR using the specific primers for each reaction and the commercial mix, PCR Master Mix (*Promega, Madison, WI. U.S.A.*)[202]. The general conditions of the reactions are shown in figure 14.

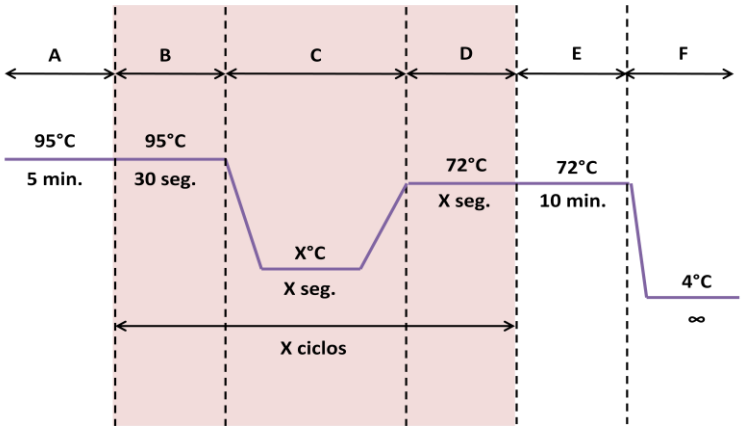

Figure 14: Scheme of the thermocycler program for the amplification of the DNA fragments by PCR. A: initial denaturation; B: initial denaturation of the cycle; C: nucleotides annealing; D: extension; E: final extension; F: PCR product preservation. Temperatures of each step are included and the number of the BCD cycles.

Every PCR product was separated by size of electrophoresis in 2% horizontal agarose gels.

2.2 MUTATION DETECTION BY CSGE-HETERODUPLEX

PCR products were denatured at 95ºC for 5 minutes and cooled at a rate of 1ºC per minute until 32ºC to facilitate heteroduplex formation. The electrophoresis was kept in vertical MDE gels for 21 hours. At the end of the session, they were dyed with the commercial DNA Silver Stainning Kit (*GE Healthcare Bio-Science AB, Uppsala, Sweden*) in order to see every band. Abnormal migration patterns of the PCR products were subsequently analyzed by automatic Sanger sequencing, prior purification procedure, to search for DNA alterations (figure 15).

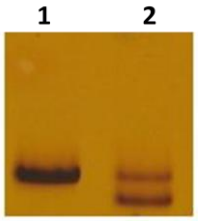

Figure 15: Example of two samples that show different patterns after applying the CSGE-Heteroduplex technique. The sample 1 shows a normal migration pattern while the simple 2 has a mutation that shows an altered pattern after the denaturation-renaturation process which forms heteroduplex and homoduplex.

2.3 BIOINFORMATICS ANALYSIS OF THE SEQUENCES

The analysis of the sequences was performed thanks to Chromas Lite program **(**http://technelysium.com.au/chromas_lite.html) and FASTA and BLAST programs (www2.ebi.ac.uk/fasta3 and www.genome.ad.jp/SIT/SIT.html respectively).

2.4 DATABASES

The databases used for all research relating to studied genes, proteins, mutations, and polymorphisms described and undescribed were: Pubmed (www.ncbi.nlm.nih.gov/pubmed)**,** UCSC (genome.ucsc.edu)[203]**,** VEGA (vega.sanger.ac.uk/index.html)**,** HGMD (www.hgmd.cf.ac.uk/ac/index.php)**,** LOVD-INSIGHT (www.insight-group.org/mutations)**,** COSMIC (www.sanger.ac.uk/genetics/CGP/cosmic)**,** IARC TP53 database (p53.iarc.fr/)**,** The P53 Web Site (p53.fr/) and Mismatch Repair Gene Variant database (www.med.mun.ca/mmrvariants/default.aspx)**.** miRBASE database (http://www.mirbase.org/) was consulted to check the possible altered miRNA binding sequences.

3. MUTATION CHARACTERIZATION

3.1 ALLELIC PERTENENCE STUDIES

When more than one mutation appeared in the same exon of the same gene or in the case of mutations located at the beginning or at the end of a sequence, the PCR fragment was cloned in pGEM®-T easy vector (*Promega, Madison, WI, U.S.A.*) (figure 16). The constructions were transformed in DH5α bacteria derived from *Escherichia coli*. After the growth incubation period at 37ºC, the selected colonies were yield for increasing the vector quantity, which was extracted by the commercial kit DanaPlasmi Spin Miniprep Kit (GeneDan S.L., Barcelona, Spain). The cloned sequences were analyzed by digestion with ApaI and SacI restriction enzymes and then followed by automatic sequencing.

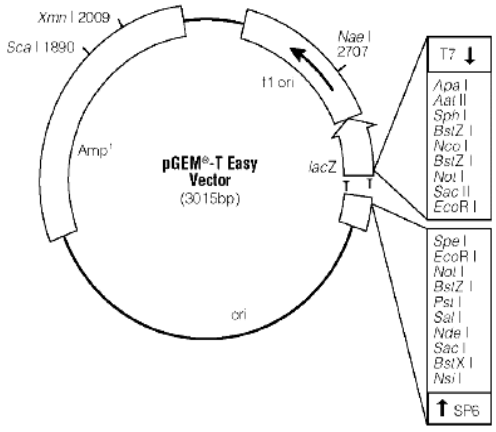

Figure 16: Scheme of the pGEM®-T easy cloning vector. The restriction sites for the enzymes and the functional sequences of reference are represented

3.2 PROGRAMS USED FOR THE UNDESCRIBED MUTATIONS *IN SILICO* STUDIES

The mutations in the *in silico* studies were applied and not reported in previous works. The informatics programs used for this analysis were PolyPhen[204,205], SIFT[206], Pmut[207], Panther[208,209]. and GVGD[210]. All of programs predicted the theoretical behaviour of the variants comparing the alignment with other related proteins or the secondary structure of the protein. PolyPhen also displayed the preservation of the changed amino acid through species. The MicroRNA program was used to predict miRNA annealing in 5´and 3´-UTR altered regions[211]. ESEfinder[212,213] 3.0 and RESCUE-ESE 1.0 were useful for checking if synonymous nucleotide changes altered splicing protein binding. PSIPRED was used towards the study of possible alterations in secondary structure of the proteins prediction when there were insertions or

deletions in the sequence[214]. Finally, TFSEARCH 1.3 allowed us to look for transcription factors which bind to promoter sequences[215].

3.3 RT-PCR

RT-PCR reaction was carried out to check possible alterations in mRNA splicing when an intronic-exonic delection in *ARID1A* occurred.

Inverse transcription and PCR reactions were done in several steps. First, cDNA was synthesized using *ImProm-II*™ *Reverse Transcription System* kit (*Promega, Madison, WI, U.S.A.*) and following manufacturer instructions. Finally, to check a proper cDNA synthesis we amplified by PCR a fragment from *GAPDH* gene, constitutionally expressed in every tissue.

3.4 Protein Expression Analysis By Western blot

Protein expression was studied in those samples where undescribed mutations were present in order to see if they were causing any pathogenic effect upon the translation or transcription mechanisms. We also studied by Western blot the expression of HDAC1, HDAC2, and HDAC3 in every sample we had.

We used four cell lines from endometrial carcinoma as controls: HEC1B, KLE, AN3CA, and SKUT1.

The loading control in the assay was the detection of β-actin protein. All the antibodies and conditions used in this study are shown (table 3):

Table 3: Antibodies used in the study of protein expression by Western blot, which is indicated the origin, the dilution of use and the manufacturer.

Antibody type	Antibody	Origin	Dilution	Manufacturer
Primary	Anti-PTEN	Mouse	1:200	Santa Cruz Biotech
	Anti-PIK3CA	Rabbit	1:1000	Abcam
	Anti-PPP2R1A	Mouse	1:5000	Abcam
	Anti-β-actina	Mouse	1:10.000	Sigma-Aldrich
	Anti-HDAC1	Rabbit	1:2000	Abcam
	Anti-HDAC2	Rabbit	1:2000	Abcam
	Anti-HDAC3	Rabbit	1:2000	Abcam
Secondary	Anti-Mouse	Sheep	1:5000-1:10.000	Sigma-Aldrich
	Anti-Rabbit	Goat	1:10.000-1:12.000	Chemicon International

3.5 MMR GENES EXPRESSION ANALYSIS BY IMMUNOCHEMISTRY

Immunochemical analysis of MMR genes were carried out in the Pathologic Anatomy Service of the University Hospital of Salamanca. In the beginning of the study this technique was not carried out, and because of this we only have the immunochemical information since case 42.

Paraffin slides were incubated with the appropriate antibodies for *hMLH1*, *hMSH2,* and *hMSH6* expression visualization (table 4).

Table 4: Primary and secondary antibodies used in the specific immunodetection of the HDACs proteins and the concentrations applied for each one.

Antibody	Dilution	Manufacturer
Anti-hMLH1	1:20	Becton Dickinson
Anti-hMSH2	Prediluted	Biocare Medical
Anti-hMSH6	Prediluted	Biocare Medical

4. LOSS OF HETEROZIGOSITY (LOH)

In order to identify the presence of both alleles in samples that showed two or more homozygous mutations in the same PCR fragment, we performed different techniques depending on the gene studied.

In the case of the *PTEN* gene, we studied the frequent polymorphism IVS4+109ins5 located in its intron 4. The assay was carried out by Restriction Fragment Length Polymorphism (RFLP) studies. The intron 4 was amplified and the product was digested with the restriction enzyme AflII (BspTI) in order to check for the possible heterozygosity of the samples. If the polymorphism was homozygous, we analyzed the LOH by quantitative comparative PCR as well as study the presence of both alleles in *TP53* and *ARID1A* genes. For this method, two DNA fragments were amplified at the same time. One of them was the fragment likely to have the gross alteration and the second was an amplification of a one-copy reference gene (*36b4* in our case) used to measure and compare the initial quantity of the total DNA from the sample. Furthermore, a sample without alterations in the genes was used as a negative control to compare the quantity levels of the amplifications with the samples of the tumours. We amplified exons 6 and 7 of *TP53*, exon 17 of *ARID1A*, and exon 11 of the *BRAF* genes. The reactions were performed with Syber Green® PCR Master Mix (*Roche, Basilea, Switzerland*) as the fluorescent agent and each reaction were done in triplicate to minimize the concentration variability of the samples.

The general conditions of the reactions are shown in figure 17.

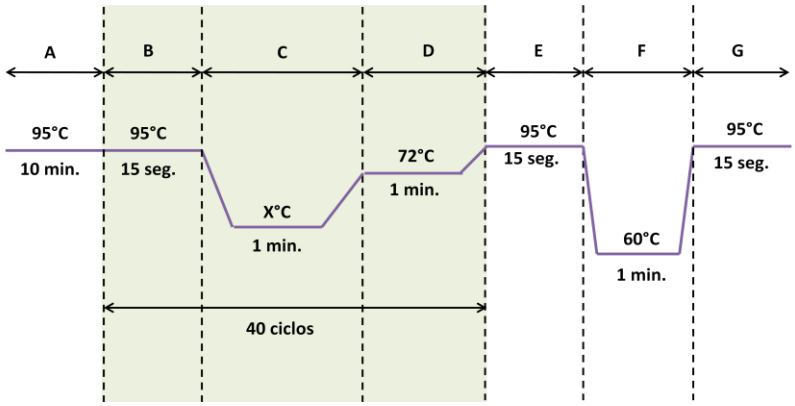

Figure 17: Scheme of the thermocycler program for the relative quantification of the DNA fragments by real time PCR. A: initial denaturation; BCD: denaturation, oligonucleotide annealing and elongation steps repeated for 40 cycles; EFG: melting program. Temperatures of each step are included.

5. MICROSATELLITE INSTABILITY STUDY

MSI analysis was possible only when we had DNA from tumour and peripheral blood samples from the same patient so it was performed in 28 of our cases. We used 8 microsatellite markers. 5 of the markers were included in Bethesda recommendations (BAT25, BAT26, D5S346, D2S123, and D17S250)[141] and the other three were used in other endometrial carcinoma MSI assays (BAT40, PAX6, and MYCL1). The conditions for the PCR amplifications were the same in every marker and are shown in figure 18.

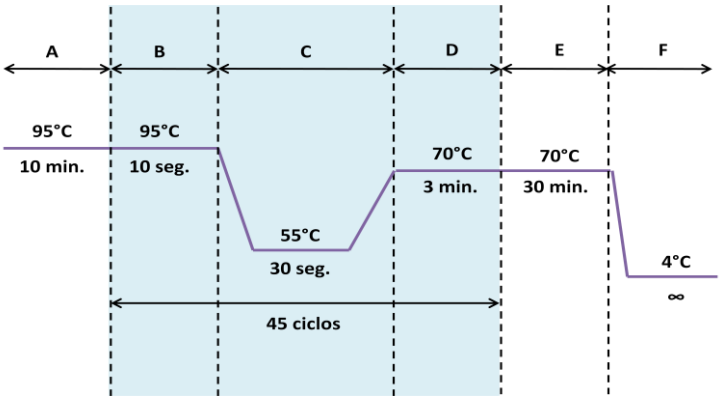

Figure 18: Scheme of the thermocycler program for the amplification of the DNA fragments by PCR in the MSI study. A: initial denaturation; B: initial denaturation of the cycle; C: nucleotides annealing; D: extension; E: final extension; F: PCR product preservation. Temperatures of each step are included and the number of the BCD cycles.

There was microsatellite instability of the peak and size pattern of the microsatellite when comparing DNA from different tumour and peripheral blood[216].

6. ANALYSIS OF GROSS GENOMIC ALTERATIONS IN MMR GENES

6.1 MLPA (MULTIPLEX LIGATION-DEPENDENT PROBE AMPLIFICATION)

hMLH1, hMSH2, and *hMSH6* genes were also analyzed for gross deletions and amplifications by MLPA assay[217]. *hMLH1* and *hMSH2* genes were analyzed from cases 1 to 41. However, from case 42, MLPA studies were carried out only in those with negative immunochemical for *hMLH1* and *hMLH2* as well as *hMSH6*.

We used two commercial kits, one for the *hMLH1* and *hMSH2* study (SALSA P003 MLH1/MSH2) and another one for the *hMSH6* analysis (SALSA P072 MSH6). Whenever possible, we included the DNA sample from the peripheral blood study. The conditions used were those recommended by the manufacturer (*MCR-Holland, Amsterdam, Holland*).

Real time quantitative comparative PCR was established in order to validate the results obtained by MLPA assay. The one-copy reference gene was *36b4*.

6.2 MS-MLPA (METHYLATION SPECIFIC-MULTIPLEX LIGATION-DEPENDENT PROBE AMPLIFICATION)

Status methylation of the promoter regions of *hMLH1*, *hMSH2,* and *hMSH6* genes was analyzed by the MS-MLPA assay[218]. This method is a variation of the MLPA with HhaI endonuclease digestion of the unmethylated DNA located in the probe-annealing site.

This study was carried out with the commercial kit SALSA ME011-B1 MMR (*MCR-Holland, Amsterdam, Holand*) in the 86 samples of tumoral DNA. The conditions used were those recommended by the manufacturer.

7. TELOMERE LENGTH MEASUREMENT BY COMPARATIVE QUANTITATIVE PCR

Telomere length measurement was carried out in collaboration with the Oncogenetics team of the Institute of Cancer Research (Sutton, United Kingdom) headed by Professor Mrs. Rosalind Eeles.

The technique used was comparative quantitative relative real time PCR. The endogenous control gene chosen was the 36b4 gene. The method used was performed by Cawton[219]. The PCRs had an efficiency of 75.45% for *36b4* gene amplification and 75.45% for the telomeres amplification.

The DNA concentration used was 10ng DNA/reaction and the reagent used was Kapa™ Syber® Fast qPCR Kit Master Mix (2X) ABI Prism (*Kappa Biosystems, Massachusetts, U.S.A.*). The conditions for the PCR amplifications are shown in figure 19.

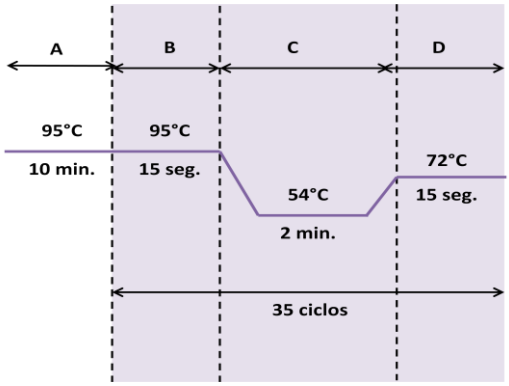

Figure 19: Scheme of the thermocycler program for the amplification of the telomeres by real time PCR. A: initial denaturation; BCD: denaturation, oligonucleotide annealing, and elongation steps repeated for 35 cycles. Temperatures of each step are included

8. *TERT-1327C>T* AND *TERC-63G>A* POLYMORPHISMS STUDY BY REAL TIME PCR.

TERT-1327C>T (rs2735940, C_1839086_10) and *TERC-63G>A* (rs2293607, C_16184740_10) polymorphisms have also been analyzed by real time PCR allelic discrimination. Both polymorphisms were studied in order to see if an association existed between groups with shorter telomeres due to the fact that they are involved with a lower activity of the telomerase[220,221]. The amplification program for both polymorphisms had an annealing temperature of 60ºC lasting one minute and the total number of 40 cycles.

9. STATISTICAL ANALYSIS

The statistical analysis for the comparison of frequencies among each molecular factor, alterations, and genotypes was carried out with the help of the SPSS v.18.0 program. GenEX 5.3.6 Enterprise was used for the telomere length measurement assay and MULTBiplot for the multivariate Biplot analysis.

RESULTS

1. Study Of Genes Associated With Sporadic Endometrial Carcinoma

In the 14 genes studied, 213 mutations were found: 99 described and 114 undescribed. The undescribed mutations were either of unknown signification or pathogenic depending on their characteristics.

Every mutation detected was analyzed from the DNA of peripherical blood samples in order to ensure that it was not inherited.

1.1 Suppressor Tumor Genes

1.1.1 Study Of *PTEN* Gene

Every change we found in *PTEN* gene are represented in the next table (table 5):

Table 5: Alterations found in the analysis of the *PTEN* gene. Pathogenic mutations already described are highlighted in red. Pathogenic mutations described by us are highlighted in yellow. Polymorphisms are not highlighted. (H): Homozygous variants.

Cases	DNA mutation	Protein mutation	Region	Type of mutation	Affected domain	Described	Function
2 and 26	c.405_406insA	p.C136fsX1	5	Insertion	Phosphatase	Yes	Pathogenic
2	c.956_959delCTTT	p.T319fsX24	8	Deletion	C2	Yes	Pathogenic
3	c.641_662del	p.Q214_K221del	7	Deletion	C2	No	Unknown*
4	c.C283A	p.P95T	5	Substitution	Phosphatase	No	Unknown*
4	c.C328T	p.Q111X	5	Substitution	Phosphatase	Yes	Pathogenic
4	c.G376A	p.A126T	5	Substitution	Phosphatase	Yes	Pathogenic
5	c.515_517delGC	p.R172fsX5	6	Deletion	Phosphatase	No	Unknown*
6	c.388_389delinsT	p.R130fsX16	5	Delins	Phosphatase	Yes	Pathogenic
7, 21, and 56	c.G389C	p.R130P	5	Substitution	Phosphatase	Yes	Pathogenic
8	IVS(6)-12insT	---	Intron 5	Insertion	---	Yes	Polymorphism
10 and 12	c.757_759delAT	p.I253fsX2	7	Deletion	C2	Yes	Pathogenic
10	c.T959C	p.L320S	8	Substitution	C2	No	Unknown*
12 and 80	c.G395A	p.G132D	5	Substitution	Phosphatase	Yes	Pathogenic
13, 18, 23, 37, 42, and 60	c.C388G	p.R130G	5	Substitution	Phosphatase	Yes	Pathogenic
14 and 22	c.G395C	p.G132A	5	Substitution	Phosphatase	Yes	Pathogenic
17	c.C743G	p.P248R	7	Substitution	C2	No	Unknown*
17	c.G752T	p.G251V	7	Substitution	C2	Yes	Pathogenic
22	c.G493A	p.G165R	6	Substitution	Phosphatase	Yes	Pathogenic
27 (H)	c.G513C	p.Q171H	7	Substitution	Phosphatase	Yes	Pathogenic
30	c.A456G	p.L152L	5	Substitution	Phosphatase	No	Unknown*
31	c.900_901insTC	p.D300fsX6	8	Insertion	C2	No	Unknown*
35 (H) and 75	c.C697T	p.R233X	7	Substitution	C2	Yes	Pathogenic
36	c.530_536delATTAT	p.Y176fsX2	6	Deletion	Phosphatase	Yes	Pathogenic
38	c.742_743insA	p.P248fsX5	7	Insertion	C2	Yes	Pathogenic
39	c.G766T	p.E256X	7	Substitution	C2	Yes	Pathogenic
44, 52, 72, 76	c.G389A	p.R130Q	5	Substitution	Phosphatase	Yes	Pathogenic

Cases	DNA mutation	Protein mutation	Region	Type of mutation	Affected domain	Described	Function
44	c.A391C	p.T131P	5	Substitution	Phosphatase	No	Unknown
47 and 54	c.968_969insAfsX1	p.N323fsX1	8	Insertion	C2	Yes	Pathogenic
48	c.C1003T	p.R335X	8	Substitution	C2	Yes	Pathogenic
51	c.G895T	p.E299X	8	Substitution	C2	No	Unknown
52	c.C424T	p.R142W	5	Substitution	Phosphatase	No	Unknown
52	c.517_518delCGinsTA	p.R173Y	6	Delins	Phosphatase	No	Unknown
56 and 60	c.G389T	p.R130L	5	Substitution	Phosphatase	Yes	Pathogenic
60	c.A474G	p.V158V	5	Substitution	Phosphatase	No	Unknown
63	c.1029_1032delGAA	p.344delK	9	Deletion	C2	No	Unknown
64 and 80	c.261_263delAT	p.Q87fsX2	5	Deletion	Phosphatase	No	Unknown
65	IVS6+1G>A	---	Intron 6	Substitution	---	Yes	Pathogenic
67 (H)	IVS3+5G>T	---	Intron 3	Substitution	---	No	Unknown
74	c.255_257delGCinsT	p.A86fsX4	5	Delins	Phosphatase	No	Unknown
76	c.G754T	p.D252Y	7	Substitution	C2	No	Unknown
78	c.955_956insA	p.T319fsX5	8	Insertion	C2	Yes	Pathogenic
82	c.G853T	p.E285X	8	Substitution	C2	No	Unknown
82	c.T202C	p.T68H	3	Substitution	Phosphatase	Yes	Pathogenic
84	c.G821A	p.W274X	8	Substitution	C2	Yes	Pathogenic
85	c.923_924delG	p.R308fsX8	8	Deletion	C2	No	Unknown
85	c.*+2insT	---	5´-ÚTR	Insertion	---	No	Unknown
86	c.G274C	p.D92H	5	Substitution	Phosphatase	No	Unknown
86	c.954_958delACTT	pT318fsX1	8	Deletion	C2	Yes	Pathogenic
86	c.T125C	p.L42P	2	Substitution	Phosphatase	No	Unknown

***These mutations were previously described in the preliminary studies carried out in our laboratory[222].**

Cases 27, 35, and 67 showed homozygous mutations without being accompanied by other heterozygous mutations. The analysis of RFLP and qPCR, however, showed that LOH was not present. Through cloning, it was observed that in tumours 4 and 17, mutations of the same exon were located in the same allele and in tumours 44, 52, 56, 60, and 86 they were located in different alleles.

We observed in tumours 45 and 79 a triple pattern of peaks in the sequence of exons 7 and 8 respectively.

p.L152L and p.V158V mutations were analyzed with ESE-finder and RESCUE-ESE programs which showed an lack of pathogenicity; to ensure these results it would be beneficial to follow-up with the mRNA study. The *In silico* studies also indicated that every missense undescribed mutation was pathogenic.

p.Q214_K221del and p.344delK inframe mutations were considered pathogenic as well because while using the PSIPRED program big alterations could be seen in the secondary structure of the protein.

For the IVS3+5G>T change study, the analysis of RNA is necessary in order to search for possible alterations in the mRNA splicing. The study of c.*+2insT using the microRNA program did not show any miRNA which could bind to the region although it would be interesting to see if RNA could cause changes in the mRNA stability.

We applied the Western blot technique for all samples which showed undescribed mutations. Only the frameshift and inframe mutations caused a decrease in the protein expression.

1.1.2 STUDY OF *TP53* GENE

Every mutation found in the *TP53* gene was previously described except one located in intron 6 (table 6).

Table 6: Alterations found in the analysis of the *TP53* gene. Pathogenic mutations already described are highlighted in red. Polymorphisms are not highlighted. (H): Homozygous variants.

Cases	DNA mutation	Protein mutation	Region	Type of mutation	Affected domain	Described	Function
3, 4, 20 (H), and 60	c.A639T	p.R213R	6	Substitution	DNA binding	Yes	Polymorphism
9	c.C665T	p.P222L	6	Substitution	DNA binding	Yes	Polymorphism
9	c.C328T	p.R110C	4	Substitution	DNA binding	Yes	Pathogenic
9	c.C916T	p.R306R	8	Substitution	DNA binding/Tetramerization	Yes	Polymorphism
11 (H)	c.C722T	p.S241F	7	Substitution	DNA binding	Yes	Pathogenic
16	c.527_528insC	p.C176fsX180	5	Insertion	DNA binding	Yes	Pathogenic
20 (H)	c.G818A	p.R273H	8	Substitution	DNA binding	Yes	Pathogenic
26, 29, and 73	c.G744A	p.R248Q	7	Substitution	DNA binding	Yes	Pathogenic
27	c.458_459insG	p.P153fsX179	5	Insertion	DNA binding	Yes	Pathogenic
27	c.C832T	p.P278S	8	Substitution	DNA binding	Yes	Pathogenic
29 and 35	c.G108A	p.P36P	4	Substitution	Transactivation	Yes	Polymorphism
32	c.T821C	p.V274A	8	Substitution	DNA binding	Yes	Pathogenic
48	c.G796A	p.G266R	8	Substitution	DNA binding	Yes	Pathogenic
52	c.C523T	p.R175C	5	Substitution	DNA binding	Yes	Pathogenic
53	c.A715G	p.N239D	7	Substitution	DNA binding	Yes	Pathogenic
57	c.C451T	p.P151S	5	Substitution	DNA binding	Yes	Pathogenic
58	c.C637T	p.R213X	6	Substitution	DNA binding	Yes	Pathogenic
59	IVS6+2T>C	---	Intron 6	Substitution	---	No	Unknown
62	c.C668A	p.A223D	5	Substitution	DNA binding	Yes	Pathogenic
70	c.A639G	p.R213R	6	Substitution	DNA binding	Yes	Polymorphism
70	c.G524A	p.R175H	5	Substitution	DNA binding	Yes	Pathogenic
70	c.G796C	p.G266R	8	Substitution	DNA binding	Yes	Pathogenic
79	c.G404A	p.C135Y	5	Substitution	DNA binding	Yes	Pathogenic
84	c.G747T	p.R249S	7	Substitution	DNA binding	Yes	Pathogenic

Through qPCR it was observed in tumour 11 that there potentially could be a loss of one allele whereas in tumour 20 both of alleles were presented.

Regarding the study of IVS6+2T>C, it would be necessary to carry out a RNA analysis in order to see if the change alters mRNA splicing.

1.1.3 STUDY OF *CDKN2A* GENE

Only 7 samples showed mutations in the *CDKN2A* gene and only one was previously described (table 7).

Table 7: Alterations found in the analysis of the *CDKN2A* gene. Pathogenic mutations described by us are highlighted in yellow. Polymorphisms are not highlighted.

Cases	DNA mutation	Protein mutation	Region	Type of mutation	Described	Function
3	IVS(2)-53T>G	---	Intron 1	Substitution	No	Unknown*
4 and 62	c.G445A	p.A148T	2	Substitution	Yes	Polymorphism
9	c.G217A	p.A73T	2	Substitution	No	Unknown*
24, 26, and 56	IVS1+37G>C	---	Intron 1	Substitution	No	Unknown*

***These mutations were previously described in the preliminary studies carried out in our laboratory[222].**

Changes in the positions of -53 y +37 in regards to exons 2 and 1 respectively were considered benign because they were distant from the exons.

It was determined through three of the five *in silico* programs used that p.A73T was pathogenic.

1.1.4 STUDY OF *CDH1* GENE

CDH1 gene analysis showed 5 polymorphisms previously described and 7 undescribed changes. Two of them could possibly be pathogenic because they were missense alterations, therefore their analysis was carried out using the *in silico* programs (table 8).

Table 8: Alterations found in the analysis of the *CDH1* gene. Polymorphisms are not highlighted.

Cases	DNA Mutation	Protein Mutation	Region	Type of mutation	Affected domain	Described	Function
18	c.C933G	p.L311L	7	Substitution	Extracellular	Yes	Polymorphism
18	c.G253A	p.V85I	3	Substitution	Precursor	No	Unknown*
22	c.C1896T	p.H632H	12	Substitution	Extracellular	Yes	Polymorphism
29	c.G271A	p.R90Q	3	Substitution	Precursor	No	Unknown*
42, 48, 61, 72, 82, and 85	IVS1+6C>T	---	Intron 1	Substitution	---	No	Unknown
45, 55, and 71	IVS4+10G>C	---	Intron 4	Substitution	---	No	Unknown
42, 44, 50, 57, 79, 61, and 78	IVS(14)-13T>C	---	Intron 13	Substitution	---	No	Unknown
72	c.C2253T	p.N715N		Substitution		Yes	Polymorphism
72, 80, and 85	c.-71C>G	---	5´-UTR	Substitution	---	Yes	Polymorphism
79	c.G2451A	p.A817A	16	Substitution	Cytoplasmatic	No	Unknown
83	c.T1845C	p.I615I	12	Substitution	Extracellular	No	Unknown
29	c.T2076C	p.A692A	16	Substitution	Cytoplasmatic	Yes	Polymorphism

***These mutations were previously described in the preliminary studies carried out in our laboratory[222].**

The prediction programs suggest that both missense mutations and p.A817A and p.I615I, analyzed by ESEfinder and RESCUE-ESE programs, were benign. More studies with RNA are now needed to check the results. Therefore, no mutations found in the *CDH1* gene were pathogenic.

1.1.5. STUDY OF *PPP2R1A* GENE

PPP2R1A gene is a fairly new gene that was included in the study of molecular profiles of endometrial carcinoma. It is normally examined in the 5th and 6th exons because pathogenic mutations have only been found in this region. We studied the whole gene in grade 3 carcinomas and only exons 5 and 6 in the rest of the samples (table 9).

Table 9: Alterations found in the analysis of the *PPP2R1A* gene. Pathogenic mutations already described are highlighted in red. Pathogenic mutations described by us are highlighted in yellow. Polymorphisms are not highlighted. (H): Homozygous variants.

Cases	DNA mutation	Protein mutation	Region	Type of mutation	Affected domain	Described	Function
9, 20, 38, 39, 59, 67, and 72	c.-52A>G	---	5´-UTR	Substitution	---	No	Unknown
9	c.-44G>C	---	5´-UTR	Substitution	---	No	Unknown
9, 20, 38, 39, 59, and 72	c.-73T>C	---	5´-UTR	Substitution	---	No	Unknown
9	c.G378A	p.A126A	4	Substitution	HEAT 4	No	Unknown
9, 59, 67, and 72	IVS6-21C>G	---	Intron 6	Substitution	---	No	Unknown
9, 67, and 72	IVS8+44A>G	---	Intron 8	Substitution	---	No	Unknown
9	IVS7+31A>G	---	Intron 7	Substitution	---	No	Unknown
9, 67, and 72	IVS11-14T>C	---	Intron 11	Substitution	---	No	Unknown
9, 59, 67, and 72	c.C1722T	p.D574D	14	Substitution	HEAT 15	No	Unknown
15, 24, 30, 50, 73, and 75	IVS5+57G>A	---	Intron 5	Substitution	---	No	Unknown
16, 19, 23, 24, 25, 27, 29, 32, 39, 70, and 80	c.-52insC	---	5´-UTR	Insertion	---	No	Unknown
16	IVS3-12T>G	---	Intron 3	Substitution	---	No	Unknown
19	IVS2-45C>T	---	Intron 2	Substitution	---	No	Unknown
20	c.-53C>T	---	5´-UTR	Substitution	---	No	Unknown
20	c.*+136C>T	---	3´-UTR	Substitution	---	No	Unknown
27 (H)	IVS2+76T>G	---	Intron 2	Substitution	---	No	Unknown
27 (H)	IVS2+87T>A	---	Intron 2	Substitution	---	No	Unknown
29	c.C767T	p.S256T	6	Substitution	HEAT 7	Yes	Pathogenic
37	c.G548A	p.R183Q	5	Substitution	HEAT 5	Yes	Pathogenic
38	c.-52insCCC	---	5´-UTR	Insertion	---	No	Unknown
45	c.G582T	p.K194N	5	Substitution	HEAT 5	No	Unknown
48, 49, 59, and 70	c.C536G	p.P179R	5	Substitution	HEAT 5	Yes	Pathogenic
52	c.G1436A	p.R48Q	2	Substitution	HEAT 2	No	Unknown
59 and 70	IVS7+42A>G	---	Intron 7	Substitution	---	No	Unknown
59	IVS14+21A>C	---	Intron 14	Substitution	---	No	Unknown
67, 73, and 80	c.-53insC	---	5´-UTR	Insertion	---	No	Unknown
70	IVS8+109C>T	---	Intron 8	Substitution	---	No	Unknown
70	c.C477T	p.S158S	4	Substitution	HEAT 4	No	Unknown
80	IVS3+31A>G	---	Intron 3	Substitution	---	No	Unknown
86	IVS7+29T>A	---	Intron 7	Substitution	---	No	Unknown

We observed three of the most frequent pathogenic mutations detected in the *PPP2R1A* gene in serous ovarian and endometrial carcinoma: p.S256T, p.R183Q, and p.P179R. We also found two undescribed missense mutations located in exon 5 and exon 2: p.K194N and p.R48Q, respectively, which were analyzed by *in silico* programs. Only p.R48Q could be considered a pathogenic mutation.

Our results showed that p.A126A, p.D574D, and p.S158S silence mutations were benign and in addition, many undescribed polymorphisms were found in intronic regions. There were a group of alterations located in the 5´-UTR region which we wanted to study using the TFSEARCH program in order to see if any transcription factors bind there: c.-52A>G (7 cases), c.-73T>C (6 cases), c.-52insC (11 cases), c.-44G>C (1 case), c.-53C>T (1 case), c.-52insCCC (1 case), and c.-53insC (3 cases). We noticed several transcription factors could bind or unbind when the 5´-UTR region was altered. For example MZF1, SP1, RUNX1/AML-1A, and IKZF1/LYF-1/IKAROS transcription factors binding was altered. We also found a miRNA which binded with a score of 75 when c.-73T>C occurred, but it was not validated by miRBASE.

We applied the Western blot technique with the purpose of seeing if the two undescribed missense mutations caused any alteration in protein expression. Although this did not occur, we did observe two patterns of bands in the developed gel: one at 65 kDa according to the weight of the protein, and another one at 110 kDa. Some of the other samples showed the same pattern and others only one of the two bands and even the HEC1B cell line presented the second unknown expression. We think it may be the result of postranslational changes in the protein.

1.1.6 Study Of *ARID1A* Gene

Most of the alterations found in the *ARID1A* gene were undescribed, frameshift, and nosense mutations. All of them were considered pathogenic. We also observed 9 pathogenic described mutations with few intronic changes (table 10).

Table 10: Alterations found in the analysis of the *ARID1A* gene. Pathogenic mutations already described are highlighted in red. Pathogenic mutations described by us are highlighted in yellow. Polymorphisms are not highlighted. (H): Homozygous variants.

Cases	DNA mutations	Protein mutations	Region	Type of mutation	Affected domain	Described	Function
1, 32, and 33	IVS4+51A>G	---	Intron 4	Substitution	---	No	Unknown
2	c.1650_1651insC	p.Y651fsX72	3	Insertion		No	Unknown
3	c.C3826T	p.R1276X	15	Substitution		Yes	Pathogenic
5, 28, and 29	IVS(8)-18G>C	---	Intron 7	Substitution	---	No	Unknown
5	c.2665_2667delG	p.G889fsX2	8	Deletion		No	Unknown
5 and 84	c.5547_5549delG	p.D1850fsX32	20	Substitution		Yes	Pathogenic
5, 12, 17, 27, 29, 39, 54, 58, 71, 72, 75, and 79	c.*+35_36insC	---	3´-UTR	Insertion	---	No	Unknown
6	c.643_645delA	p.Y215fsX17	1	Deletion		No	Unknown
10 (H)	IVS17+42C>T	---	Intron 17	Substitution	---	No	Unknown
14	c.256_286del	p.G86fsX14	1	Deletion		No	Unknown
17 and 18	IVS4+28delC	---	Intron 4	Deletion	---	No	Unknown
17	c.3056_3057insA	p.E1019fsX12	11	Insertion	DNA binding domain	No	Unknown
21	c.4689_4690insC	p.M1564fsX7	18	Insertion		Yes	Pathogenic
22	c.6747_6748insA	p.E2250fsX27	20	Insertion		No	Unknown

Cases	DNA mutations	Protein mutations	Region	Type of mutation	Affected domain	Described	Function
23	c.5334_5336delG	p.E1779fsX3	20	Deletion		No	Unknown
23	c.3977_3978insC	p.P1326fsX11	16	Insertion		No	Unknown
26	c.4001_4002dupGCA	p.Q1334_R1335dupQ	16	Insertion	Poly-Gln	Yes	Pathogenic
28	IVS17+14C>T	---	Intron 17	Substitution	---	No	Unknown
30	c.826_828delG	p.G276fsX86	1	Deletion		Yes	Pathogenic
31	c.C1348T	p.Q450X	2	Substitution		No	Unknown
35	c.2271_2273delC	p.Q758fsX75	7	Deletion		No	Unknown
36	c.4408_4012delGATT	p.D337fsX143	17	Deletion		No	Unknown
39	c.3518_3520delC	p.I1173fsX6	13	Deletion		No	Unknown
39	c.C6343T	p.Q2115X	20	Substitution		No	Unknown
40	c.2184_2189delCCCA	p.P729fsX12	6	Deletion		No	Unknown
40	c.G5717A	p.R1906Q	18	Substitution		No	Unknown
41	c.5346_5347insT	p.E1783X	20	Insertion		No	Unknown
44	c.3977_3978insC	p.P1326fsX11	16	Insertion		No	Unknown
45	c.1635_1637delC	p.Q546fsX72	3	Deletion		No	Unknown
45	c.C4582T	p.R1528X	18	Substitution		Yes	Pathogenic
45	c.G3327A	p.R1109R	12	Substitution		No	Unknown
48	c.4840_4842delA	p.Q1614fsX20	18	Deletion		No	Unknown
52	c.G4610A	p.G1549D	18	Substitution		No	Unknown
54	IVS16+35C>T	---	Intron 16	Substitution	---	No	Unknown
55	c.G5329T	p.E1776X	20	Substitution		No	Unknown
58	c.C2999A	p.S1000Y	11	Substitution	Poly Ser	No	Unknown
60	c.C4336T	p.R1446X	18	Substitution		Yes	Pathogenic
61	c.394_399del	p.V132fsX99	1	Deletion		No	Unknown
66	c.317_332del	p.N106fsX5	1	Deletion		No	Unknown
67	c.437_438insC	p.P146fsX253	1	Insertion		No	Unknown
68	c.4311_4313delA	p.T1438fsX6	18	Deletion		No	Unknown
69	c.G6273A	p.W2091X	20	Substitution		No	Unknown
75	c.1112_1114delG	p.G371fsX19	1	Deletion		No	Unknown
76	c.3343_3345delC	p.P1115fsX44	12	Deletion		Yes	Pathogenic
78	c.471_500del	p.P158fsX65	1	Deletion		No	Unknown
79	c.3523_3525delC	p.P1175fsX4	13	Deletion		Yes	Pathogenic
79	c.5590_5593delAG	p.E1864fsX35	20	Deletion		No	Unknown
80	c.3241_3243delA	p.N1081fsX11	12	Deletion	DNA binding domain	No	Unknown
82	c.6340_6342delC	p.P2114fsX20	20	Deletion		No	Unknown
85	IVS(11)-61G>T	---	Intron 10	Substitution	---	No	Unknown
86	IVS5+57G>A	---	Intron 5	Substitution	---	No	Unknown
86	g.5346_5377del	p.G1665A	Intron 18-Exon19	Deletion	---	No	Unknown

The study of LOH by qPCR in sample 10 showed that both alleles were presented.

Intronic substitutions were discarded as pathogenic because of their distant localization of the exons. c.*+35_36insC situated in 3´-UTR region was analyzed in control population observing it is a frequent change in non-affected people. Therefore, we consider it is a polymorphism.

We detected three missense mutations non-previously described: p.R1906Q, p.G1549D and p.S1000Y. Prediction programs suggested that only p.R1906Q did not affect to the function of the protein.

On the other hand, we found two inframe deletions. One of them was described several times in different kind of tumours: p.Q1334_R1335dupQ and the other one was a deletion

of 30 nucleotides affecting the intron 18 and the exon 19: g.5346_5377del with unknown effect. It was analyzed by RT-PCR program observing that the mutation causes a transcription of intron 18 and 19 but not of exon 20.

ESEfinder and RESCUE-ESE programs did not show relevant alterations in binding sites of proteins when p.R1109R occurred. We classified it as a polymorphism.

1.2 ONCOGENES

1.2.1 STUDY OF *PIK3CA* GENE

PIK3CA gene analysis showed the presence of missense mutations almost all located in exon 20 (table 11).

Table 11: Alterations found in the analysis of the *PIK3CA* gene. Pathogenic mutations already described are highlighted in red. Pathogenic mutations described by us are highlighted in yellow. Polymorphisms are not highlighted.

Cases	DNA mutation	Protein mutation	Region	Type of mutation	Affected domain	Described	Function
3	c.1353_1380del	p.G451_460del	7	Deletion	C2	No	Unknown*
8	c.T3061C	p.Y1021H	20	Substitution	Kinase	Yes	Pathogenic
9	c.C2974T	p.R992X	20	Substitution	Kinase	No	Unknown*
10, 46, and 82	c.A3127G	p.M1043V	20	Substitution	Kinase	Yes	Pathogenic
12	c.T3132G	p.N1044K	20	Substitution	Kinase	Yes	Pathogenic
22 and 31	c.A3140G	p.H1047R	20	Substitution	Kinase	Yes	Pathogenic
40	c.G1635T	p.E545D	9	Substitution	Helix	Yes	Pathogenic
42	c.A3194T	p.H1065L	20	Substitution	Kinase	Yes	Pathogenic
50 and 52	c.A3073G	p.T1025A	20	Substitution	Kinase	Yes	Pathogenic
52	c.G3072T	p.K1024N	20	Substitution	Kinase	No	Unknown
58	c.G3129T	p.M1043I	20	Substitution	Kinase	Yes	Pathogenic
65	IVS20+29C>T	---	Intron 20	Substitution	---	No	Unknown
73	IVS9+17T<G	---	Intron 9	Substitution	---	No	Polimorfismo
78	c.G3166A	p.D1056N	20	Substitution	Kinase	No	Unknown
78	c.A3172T	p.I1058F	20	Substitution	Kinase	No	Unknown
78	c.*+5A<C	---	3´-UTR	Substitution	---	No	Unknown

***These mutations were previously described in the preliminary studies carried out in our laboratory[222].**

For the 8 undescribed mutations we carried out the same procedures already described for the rest of the genes and our results suggested that only p.G451_460del, p.R992X, p.K1024N, and p.D1056N were pathogenic.

Afterwards, we carried out a Western blot assay in order to see if mutations considered pathogenic caused any alterations in the *PIK3CA* protein expression; we noticed that p.R992X induced the absence of the expression while p.K1024N (plus pathogenic p.T1025A mutation) caused only a reduction in the *PIK3CA* expression. The rest of the changes did not provoke any alteration in the protein expression.

1.2.2 STUDY OF *CTNNB1* GENE

The analysis of the *CTNNB1* gene showed previously described pathogenic mutations in 13 tumours, one of them had two alterations in different alleles. All of them were substitutions affecting the regulatory domain (table 12).

Table 12: Alterations found in the analysis of the *CTNNB1* gene. Pathogenic mutations already described are highlighted in red.

Cases	DNA mutation	Protein mutation	Region	Type of mutation	Affected domain	Described	Function
4	c.G94A	p.D32N	3	Substitution	Regulatory	Yes	Pathogenic
10, 31, 49, 50, and 68	c.C111T	p.S37F	3	Substitution	Regulatory	Yes	Pathogenic
12 and 24	c.A122G	p.T41A	3	Substitution	Regulatory	Yes	Pathogenic
22, 34, and 68	c.G101A	p.G34R	3	Substitution	Regulatory	Yes	Pathogenic
41	c.C98G	p.S33C	3	Substitution	Regulatory	Yes	Pathogenic
56	c.C134T	p.S45F	3	Substitution	Regulatory	Yes	Pathogenic
69	c.C111G	p.S37F	3	Substitution	Regulatory	Yes	Pathogenic

1.2.3 STUDY OF *KRAS* GENE

When we studied the *KRAS* gene we observed 5 already described pathogenic mutations in 14 tumours. All of them were substitutions affecting the effector domain and located in codons 12 and 13. No mutations were found in codon 61 (table 13).

Table 13: Alterations found in the analysis of the *KRAS* gene. Pathogenic mutations already described are highlighted in red.

Cases	DNA mutation	Protein mutation	Region	Type of mutation	Affected domain	Described	Function
15, 28, 38, and 65	c.G35T	p.G12V	2	Substitution	Effector	Yes	Pathogenic
23, 35, 67, and 68	c.G38A	p.G13D	2	Substitution	Effector	Yes	Pathogenic
34, 45, 54, 60, and 82	c.G35A	p.G12D	2	Substitution	Effector	Yes	Pathogenic
80	c.G35C	p.G12A	2	Substitution	Effector	Yes	Pathogenic

1.2.4 STUDY OF *BRAF* GENE

The *BRAF* study showed a pathogenic homozygous exonic mutation in one tumour. There was no trace of LOH. The other mutation was considered a polymorphism because of its far location from the other exons (table 14).

Table 14: Alterations found in the analysis of the *BRAF* gene. Pathogenic mutations already described are highlighted in red. Polymorphisms are not highlighted. (H): Homozygous variants.

Cases	DNA mutation	Protein mutation	Region	Type of mutation	Affected domain	Described	Function
8	IVS15+17delT	---	Intron 15	Deletion	---	No	Unknown
73 (H)	c.G1407C	p.G469A	11	Substitution	Kinase	Yes	Pathogenic

1.2.5 STUDY OF *EGFR* GENE

The results of the *EGFR* gene study were negative for pathogenic mutations. We only observed two polymorphisms. p.Q787Q was found in 69 cases and was an alteration frequently present in the different types of tumours. IVS18+19G>A was found only in two tumours but the change was far from exon 18 (table 15).

Table 15: Alterations found in the analysis of the *EGFR* gene. Polymorphisms are not highlighted.

Cases	DNA mutation	Protein mutation	Region	Type of mutation	Affected domain	Described	Function
69 cases	c.G2361A	p.Q787Q	20	Substitution	Kinase	Yes	Polymorphism
53 and 76	IVS18+19G>A	---	Intron 18	Substitution	---	No	Unknown

1.3 MMR GENES

In cases 1 through 41 we performed the analysis of *hMLH1* and hMSH2 genes in every tumour. In a second trial we analyzed cases 42 to 86 only when they showed negative immunohistochemical for *hMLH1, hMSH2,* and *hMSH6* proteins.

1.3.1 STUDY OF *HMLH1* GENE

All the alterations detected in the *hMLH1* gene were already described and two of them were pathogenic (table 16).

Table 16: Alterations found in the analysis of the *hMLH1* gene. Pathogenic mutations already described are highlighted in red. Polymorphisms are not highlighted.

Cases	DNA mutation	Protein mutation	Region	Type of mutation	Affected domain	Described	Function
6, 25, and 55	c.G1959T	p.L653L	17	Substitution	EXO1 interacting	Yes	Polymorphism
8	c.1852_1853AA>GC	p.K618A	16	Substitution	EXO1 interacting	Yes	Pathogenic
30	IVS3+5G>A	---	Intron 3	Substitution	---	Yes	Pathogenic
48, 67, 82, and 84	c.A655G	p.I219V	8	Substitution	Structural	Yes	Polymorphism

Because blood samples from patients 8 and 30 were not available, we could not check if these mutations were inheritated, associated to Lynch syndrome, or somatic.

1.3.2 STUDY OF *HMSH2* GENE

In *hMSH2* we found two described polymorphisms and one mutation with an unknown function (table 17).

Table 17: Alterations found in the analysis of the *hMHS2* gene. Polymorphisms are not highlighted.

Cases	DNA mutation	Protein mutation	Region	Type of mutation	Affected domain	Described	Function
9	c.G460A	p.A154T	3	Substitution	Structural	No	Unknown*
24	IVS(13)-6T>C	---	Intron 12	Substitution	---	Yes	Polymorphism
13	IVS1+9C>G	---	Intron 1	Substitution	---	Yes	Polymorphism

***This mutation was previously described in the preliminary studies carried out in our laboratory[222].**

Due to the results obtained from the *in silico* study we categorized the undescribed mutation as being a polymorphism, but bcause we did not have the sample we could not check if the alteration was in the peripherical blood.

1.3.3 STUDY OF *HMSH6* GENE

We analyzed the *hMSH6* gene in those tumours who had negative immunohistochemical results for the *hMSH6* protein and all results obtained were negative. We did not detect mutations or polymorphisms.

1.4 GLOBAL ANALYSIS OF THE MUTATIONAL STUDY

It can be observed in the following table the number of pathogenic mutations found as well as the number of pathogenic mutations we had after characterizing the non-previously described mutations (table 18).

Table 18: Summary of the number of pathogenic mutations described before and after the characterization carried out in our study and number of mutated tumour for each analyzed gene.

TYPE	GENE	NUMBER OF MUTATIONS		NUMBER OF TUMOURS AFFECTED
		DESCRIBED PATHOGENIC MUTATIONS	TOTAL NUMBER OF PATHOGENIC MUTATIONS*	
TUMOUR SUPPRESSORS	*PTEN*	28	46	44 (51.16%)
	TP53	21	21	18 (20.93%)
	CDKN2A	0	1	1 (1.16%)
	CDH1	0	0	0 (0.00%)
	PPP2R1A	3	4	7 (8.14%)
	ARID1A	9	41	37 (43.02%)
ONCOGENES	*PIK3CA*	8	12	15 (17.44%)
	CTNNB1	7	7	13 (15.12%)
	KRAS	4	4	14 (16.28%)
	BRAF	1	1	1 (1.16%)
	EGFR	0	0	0 (0.00%)
MMR GENES	*hMLH1*	1	1	2 (2.32%)
	hMSH2	0	0	0 (0.00%)
	hMSH6	0	0	0 (0.00%)

*** After carrying out our analysis.**

PTEN and *ARID1A* genes were the most frequently altered genes considering that they normally show more than one mutation per tumour. The study is segmented according to the clinical-pathological and histological classifications we obtained (table 19):

Table 19: Percentage of mutated tumours in each gene depending on the type of tumour according to the clinical-pathological classification (type 1 or endometrioids and type 2 or non-endometrioids), histological grade (grade 1, 2, and 3) and histological subtypes (grade 1 endometrioids, grade 2 endometrioids, grade 3 endometrioids, serous, clear cells, mixed and carcinosarcoma). E1: grade 1 endometrioid carcinoma; E2: grade 2 endometrioid carcinoma; E3: grade 3 endometrioid carcinoma; C.C.: clear cells endometrial carcinoma; CR: carcinosarcoma.

TUMOUR	ALTERED GENES													
	PTEN	*TP53*	*CDKN2A*	*CDH1*	*PPP2R1A*	*ARID1A*	*PIK3CA*	*CTNNB1*	*KRAS*	*BRAF*	*EGFR*	*hMLH1*	*hMSH2*	*hMSH6*
Type 1	38 (60.32%)	8 (12.70%)	1 (1.59%)	0 (0.00%)	3 (4.76%)	30 (47.62%)	13 (20.63%)	11 (17.46%)	13 (20.63%)	1 (1.59%)	0 (0.00%)	1 (1.59%)	0 (0.00%)	0 (0.00%)
Type 2	6 (23.09%)	10 (43.48%)	0 (0.00%)	0 (0.00%)	4 (17.39%)	7 (30.43%)	2 (8.69%)	2 (8.69%)	1 (4.35%)	0 (0.00%)	0 (0.00%)	1 (4.35%)	0 (0.00%)	0 (0.00%)
G 1	18 (58.06%)	3 (9.68%)	0 (0.00%)	0 (0.00%)	1 (3.23%)	11 (35.48%)	8 (25.81%)	8 (25.81%)	4 (12.90%)	1 (3.23%)	0 (0.00%)	1 (3.23%)	0 (0.00%)	0 (0.00%)
G 2	14 (58.33%)	3 (12.50%)	0 (0.00%)	0 (0.00%)	1 (4.17%)	14 (58.33%)	2 (8.33%)	3 (12.50%)	5 (20.83%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
G 3	12 (38.71%)	12 (38.71%)	1 (3.23%)	0 (0.00%)	5 (16.13%)	12 (38.71%)	5 (16.13%)	2 (6.45%)	5 (16.13%)	0 (0.00%)	0 (0.00%)	1 (3.23%)	0 (0.00%)	0 (0.00%)
E1	18 (58.06%)	3 (9.68%)	0 (0.00%)	0 (0.00%)	1 (3.23%)	11 (35.48%)	8 (25.81%)	8 (25.81%)	4 (12.90%)	1 (3.23%)	0 (0.00%)	1 (3.23%)	0 (0.00%)	0 (0.00%)
E2	14 (58.33%)	3 (12.50%)	0 (0.00%)	0 (0.00%)	1 (4.17%)	14 (58.33%)	2 (8.33%)	3 (12.50%)	5 (20.83%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
E3	6 (75.00%)	2 (25.00%)	1 (12.50%)	0 (0.00%)	1 (12.50%)	5 (62.50%)	3 (37.50%)	0 (0.00%)	4 (50.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Serous	0 (0.00%)	4 (50.00%)	0 (0.00%)	0 (0.00%)	4 (50.00%)	0 (0.00%)	0 (0.00%)	1 (12.50%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
C.C	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	1 (100.0%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	1 (100.0%)	0 (0.00%)	0 (0.00%)
Mixed	1 (50.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	2 (100.0%)	0 (0.00%)	0 (0.00%)	1 (50.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
CR	5 (41.67%)	6 (50.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	4 (33.33%)	2 (16.67%)	1 (8.33%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)

Of note that *PTEN* gene seems to be associated to endometrioid carcinoma (60.32%), especially grade 3 endometrioid (75.00%). On the other hand *TP53* is mainly altered in non-endometrioid carcinomas (43.48%). The *PPP2R1A* gene is mutated in serous carcinoma (50.00%) and *ARID1A* is altered in both endometrioid (47.62%) and non-endometrioid carcinoma (30.43%). Mutations in the *KRAS* gene are more frequent in endometrioid carcinoma and the percentage of tumours affected in this gene increases

with the grade of endometrioid tumour (E1: 12.90%, E2: 20.83%, E3: 50.00%). *CDH1*, *EGFR*, *hMSH2,* and *hMSH6* genes showed no mutations in any tumour.

Moreover, the number of altered genes per tumour was higher in endometrioid carcinomas in more cases with 3 o more mutations (22.22% vs 13.04%) and with 2 altered genes per tumour (41.47% vs 26.09%). Groups with percentages higher than 3% or with more mutations per tumour were grade 3 endometrioid (37.50%) with mixed carcinomas (50.00%). This last group was only formed by two tumours (table 20).

Table 20: Percentage of tumours with presence of 0, 1, 2, and 3 or more mutations in each type of tumour according to the clinical-pathological classification (type 1 or endometrioids and type 2 or non-endometrioids), histological grade (grade 1, 2, and 3) and histological subtypes (grade 1 endometrioids, grade 2 endometrioids, grade 3 endometrioids, serous, clear cells, mixed and carcinosarcoma). E1: grade 1 endometrioid carcinoma; E2: grade 2 endometrioid carcinoma; E3: grade 3 endometrioid carcinoma; C.C.: clear cells endometrial carcinoma; CR: carcinosarcoma.

Mutations / Type	0	1	2	3 or more
Type 1	6 (9.52%)	17 (26.98%)	26 (41.27%)	14 (22.22%)
Type 2	5 (21.74%)	9 (39.13%)	6 (26.09%)	3 (13.04%)
Grade 1	4 (12.90%)	9 (29.03%)	11 (35.48%)	7 (22.58%)
Grade 2	2 (8.33%)	7 (29.17%)	11 (45.83%)	4 (16.67%)
Grade 3	5 (16.13%)	10 (32.26%)	10 (32.26%)	6 (19.35%)
E1	4 (12.90%)	9 (29.03%)	11 (35.48%)	7 (22.58%)
E2	2 (8.33%)	7 (29.17%)	11 (45.83%)	4 (16.67%)
E3	0 (0.00%)	1 (12.50%)	4 (50.0%)	3 (37.50%)
Serous	2 (25.00%)	3 (37.50%)	3 (37.50%)	0 (0.00%)
C. C.	0 (0.00%)	0 (0.00%)	1 (100.00%)	0 (0.00%)
Mixed	0 (0.00%)	1 (50.00%)	0 (0.00%)	1 (50.00%)
CR	2 (16.67%)	5 (41.67%)	3 (25.00%)	2 (16.67%)

2. MICROSATELLITE INSTABILITY (MSI)

We carried out the MSI analysis in the previously obtained tumour and peripherical blood samples. We studied 8 markers in 28 cases. We considered microsatellite stability when no marker was instable, low-grade microsatellite instability when 1 or 2 markers were instable, and high-grade microsatellite grade when 3 or more markers were instable (table 21).

Table 21: Results of the MSI analyzed in the 28 cases of endometrial carcinoma included in our study. E: Stable; I: Instable; MSS: Microsatellite stability; MSI-L: Low-grade microsatellite instability; MSI-H: High-grade microsatellite instability.

Case	BAT25	BAT26	D5S346	D2S123	D17S250	BAT40	PAX6	MYCL1	TOTAL ALTERED	MSI
1	E	E	E	E	E	E	E	E	0	MSS
3	E	E	I	I	I	E	E	I	4	MSI-H
15	E	E	E	E	E	E	E	E	0	MSS
16	E	E	I	E	E	E	I	I	3	MSI-H
17	E	E	E	E	E	E	E	E	0	MSS
21	I	I	E	E	I	E	I	E	4	MSI-H
23	I	E	I	I	E	E	E	E	3	MSI-H
25	E	E	E	I	I	E	E	I	3	MSI-H
37	E	E	E	E	I	I	I	E	3	MSI-H
40	E	I	I	I	I	I	I	E	6	MSI-H
41	E	E	E	E	E	E	E	E	0	MSS
42	E	E	E	E	E	E	E	E	0	MSS
43	E	E	E	E	E	I	E	E	1	MSI-L
44	I	I	E	I	I	I	I	I	7	MSI-H
45	I	I	E	E	I	I	I	I	1	MSI-L
49	E	E	E	E	E	E	E	E	0	MSS
50	I	E	E	E	E	E	E	E	1	MSI-L
52	E	E	E	E	I	E	E	E	1	MSI-L
53	E	E	E	E	E	E	E	E	0	MSS
54	E	E	I	I	E	I	E	E	3	MSI-H
55	I	I	I	I	I	E	I	I	7	MSI-H
65	E	E	E	E	E	E	E	E	0	MSS
68	E	E	E	I	E	E	E	E	1	MSI-L
69	E	E	E	E	E	E	E	I	1	MSI-L
70	E	E	E	E	I	E	E	E	1	MSI-L
79	I	I	I	I	I	E	I	I	7	MSI-H
80	E	I	I	E	E	E	E	I	3	MSI-H
86	E	E	E	E	E	E	E	E	0	MSS

9 cases showed microsatellite stability (32.14%) and 19 cases microsatellite instability (67.86%): 7 with low grade instability and 12 with high grade instability.

The most instable marker was D17S250 (39.29%) and the least instable was BAT40 (21.43%).

When we subdivided the tumours in groups according to the clinical-pathological and histological classifications, we observed differences between types and grades (table 22):

Table 22: Percentage of tumours with microsatellite stability (MSS) and low-grade (MSI-L) or high-grade (MSI-H) microsatellite instability (MSI) in each type of tumour according to the clinical-pathological classification (type 1 or endometrioids and type 2 or non-endometrioids), histological grade (grade 1, 2, and 3) and histological subtypes (grade 1 endometrioids, grade 2 endometrioids, grade 3 endometrioids, serous, clear cells, mixed and carcinosarcoma). E1: grade 1 endometrioid carcinoma; E2: grade 2 endometrioid carcinoma; E3: grade 3 endometrioid carcinoma; C.C.: clear cells endometrial carcinoma; CR: carcinosarcoma.

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MSI / Type	MSS	MSI	MSI-L	MSI-H
Type 1	7 (31.82%)	15 (68.18%)	6 (27.27%)	9 (40.91%)
Type 2	2 (33.33%)	4 (66.67%)	1 (16.67%)	3 (50.00%)
Grade 1	3 (30.00%)	7 (70.00%)	3 (30.00%)	4 (40.00%)
Grade 2	4 (40.00%)	6 (60.00%)	2 (20.00%)	4 (40.00%)
Grade 3	2 (25.00%)	6 (75.00%)	2 (25.00%)	4 (50.00%)
E1	3 (30.00%)	7 (70.00%)	3 (30.00%)	4 (40.00%)
E2	4 (40.00%)	6 (60.00%)	2 (20.00%)	4 (40.00%)
E3	0 (0.00%)	2 (100.00%)	1 (50.00%)	1 (50.00%)
Serous	1 (33.33%)	2 (66.67%)	1 (33.33%)	1 (33.33%)
C. C.	---	---	---	---
Mixed	0 (0.00%)	1 (100.00%)	0 (0.00%)	1 (100.00%)
CR	1 (50.00%)	1 (50.00%)	0 (0.00%)	1 (50.00%)

No big differences existed between the instability of endometrioid and non-endometrioid carcinomas but among grades we observed that grade 3 was the most instable group. This difference was best detected when we paid close attention to the subgroups that comprised it: 100% instability existed in grade 3 endometrioid, 66.67% in serous carcinoma, 100% in mixed, and 50% in carcinosarcomas.

3. Analysis Of Other Genomic Alterations In MMR Genes

3.1 Analysis Of Gross Chromosomal Alterations In *hMLH1*, *hMSH2*, And *hMSH6* Genes.

First we studied gross chromosomal alterations in *hMLH1* and *hMSH2* genes in samples 1 through 41 because immunohistochemical analysis were not performed in these tumours. From case 42 through 86, we only studied those samples that had a negative immunohistochemical for *hMLH1*, *hMSH2*, and *hMSH6* protein expression and with no pathogenic mutation.

Cases 8 and 16 showed the amplification and deletion of *hMLH1* while case 14 contained the amplification of exons 8, 9, and 10 in the *hMSH2* gene. Case 24 presented an amplification of the whole *hMSH2* gene. All of them were checked by qPCR.

In the second phase of the study, we analyzed the *hMLH1* and *hMSH2* genes in cases 44, 45, 48, 55, 64, 67, 71, 75, 78, 82, and 84 but none of them showed any alteration. We also studied the *hMSH6* gene in case 76 that showed a possible deletion of exon 1 in both *hMSH6* and *hMLH1* genes. With the qPCR and the analysis of *hMLH1* and *hMSH2* genes by MLPA we observed that it was a false positive because no alterations were confirmed.

3.2 Study Of The Methylation Status Of The Promoter Region Of *hMLH1, hMSH2* And *hMSH6* Genes.

The analysis of the methylation status of MMR genes was carried out in all the tumours.

A total of 42 tumours (48.84%) showed at least one of the three genes methylated. Of them, 9 cases (10.47%) had the three genes methylated; 12 (13.95%), two of them: 7 *hMLH1* and *hMLH2*, 5 *hMLH1* and *hMSH6,* and none *hMSH2* and *hMSH6* at the same time; and 21 cases (24.42%) showed one of the three genes methylated: 9 (10.47%) *hMLH1*, none (0%) *hMSH2,* and 12 (13.95%) *hMSH6*. The most frequent situation was to find only the *hMSH6* gene methylated. *hMLH1* gene was methylated in 33 cases (38.37%), *hMSH2* in 13 (15.12%), and *hMSH6* in 26 (30.23%).

When we segmented the study in subtypes, we observed next results (table 23):

Table 23: Percentage of tumours with presence or absence of methylation in the *hMLH1*, *hMSH2* and *hMSH6* genes in each type of tumour according to the clinical-pathological classification (type 1 or endometrioids and type 2 or non-endometrioids), histological grade (grade 1, 2, and 3) and histological subtypes (grade 1 endometrioids, grade 2 endometrioids, grade 3 endometrioids, serous, clear cells, mixed and carcinosarcoma). E1: grade 1 endometrioid carcinoma; E2: grade 2 endometrioid carcinoma; E3: grade 3 endometrioid carcinoma; C.C.: clear cells endometrial carcinoma; CR: carcinosarcoma.

Methylation Type	Non-methylated	Methylated	Met. *hMLH1*	Met. *hMSH2*	Met. *hMSH6*
Type 1	28 (44.44%)	35 (55.56%)	28 (44.44%)	10 (15.87%)	18 (19.05%)
Type 2	14 (60.87%)	9 (39.13%)	5 (21.74%)	3 (13.04%)	8 (34.88%)
Grade 1	14 (45.16%)	17 (54.84%)	11 (35.48%)	5 (16.13%)	9 (29.03%)
Grade 2	12 (50.00%)	12 (50.00%)	12 (50.00%)	5 (20.83%)	7 (26.17%)
Grade 3	17 (54.84%)	14 (45.16%)	10 (32.26%)	3 (9.68%)	10 (32.26%)
E1	14 (45.16%)	17 (54.84%)	11 (35.48%)	5 (16.13%)	9 (29.03%)
E2	12 (50.00%)	12 (50.00%)	12 (50.00%)	5 (20.83%)	7 (26.17%)
E3	3 (37.50%)	5 (62.50%)	5 (62.50%)	0 (0.00%)	2 (25.00%)
Serous	5 (62.50%)	3 (37.50%)	2 (25.00%)	1 (12.50%)	3 (37.50%)
C. C.	1 (100.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Mixed	2 (100.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
CR	6 (50.00%)	6 (50.00%)	3 (25.00%)	2 (16.67%)	5 (41.67%)

Moreover, the number of tumours which showed lack of methylation or methylation in 1, 2, or 3 of the studied genes could be associated with the type of tumour (figures 20A, B and C).

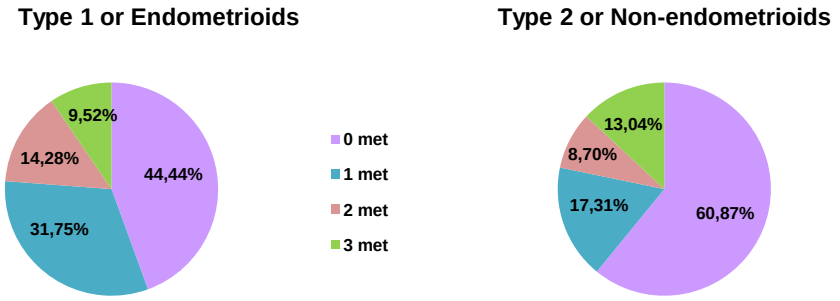

Figure 20A: **Percentage of tumours with 1, 2, 3, or none MMR genes methylated in each type of tumour according to the clinical-pathological classification.**

In non-endometrioid carcinomas is more frequent not to have any methylated gene (60.87%) but in endometrioid carcinomas the most common situation is to present only one gene methylated (31.75%). In both groups, the number of tumours with three MMR genes methylated doesn´t differ very much (9.52% and 13.04%).

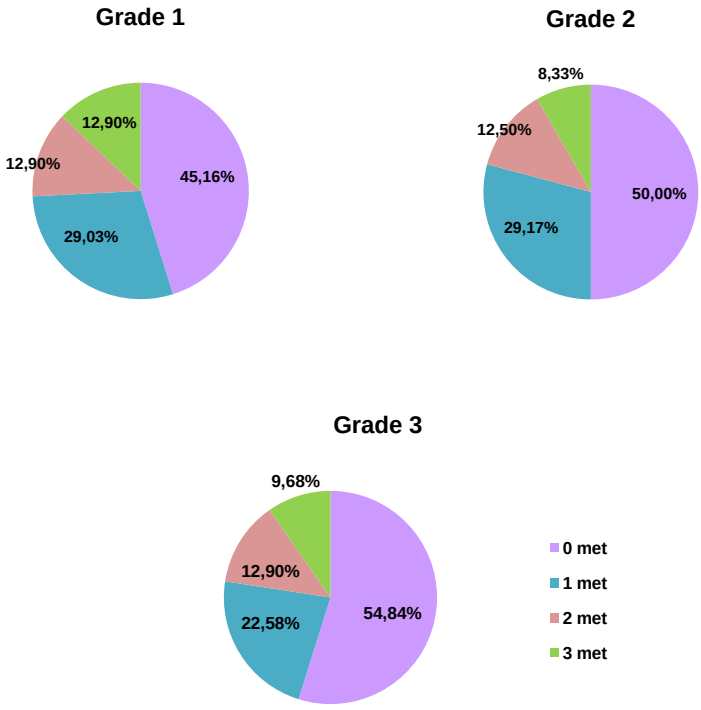

Figure 20B: Percentage of tumours with 1, 2, 3, or none MMR genes methylated in each type of tumour according to the classification by histological grades.

The percentage of tumours without any methylated gene was around the 50% being the grade 1 carcinomas those which showed a higher number of cases with *hMLH1, hMSH2*, and *hMSH6* genes methylated at the same time (12.90%).

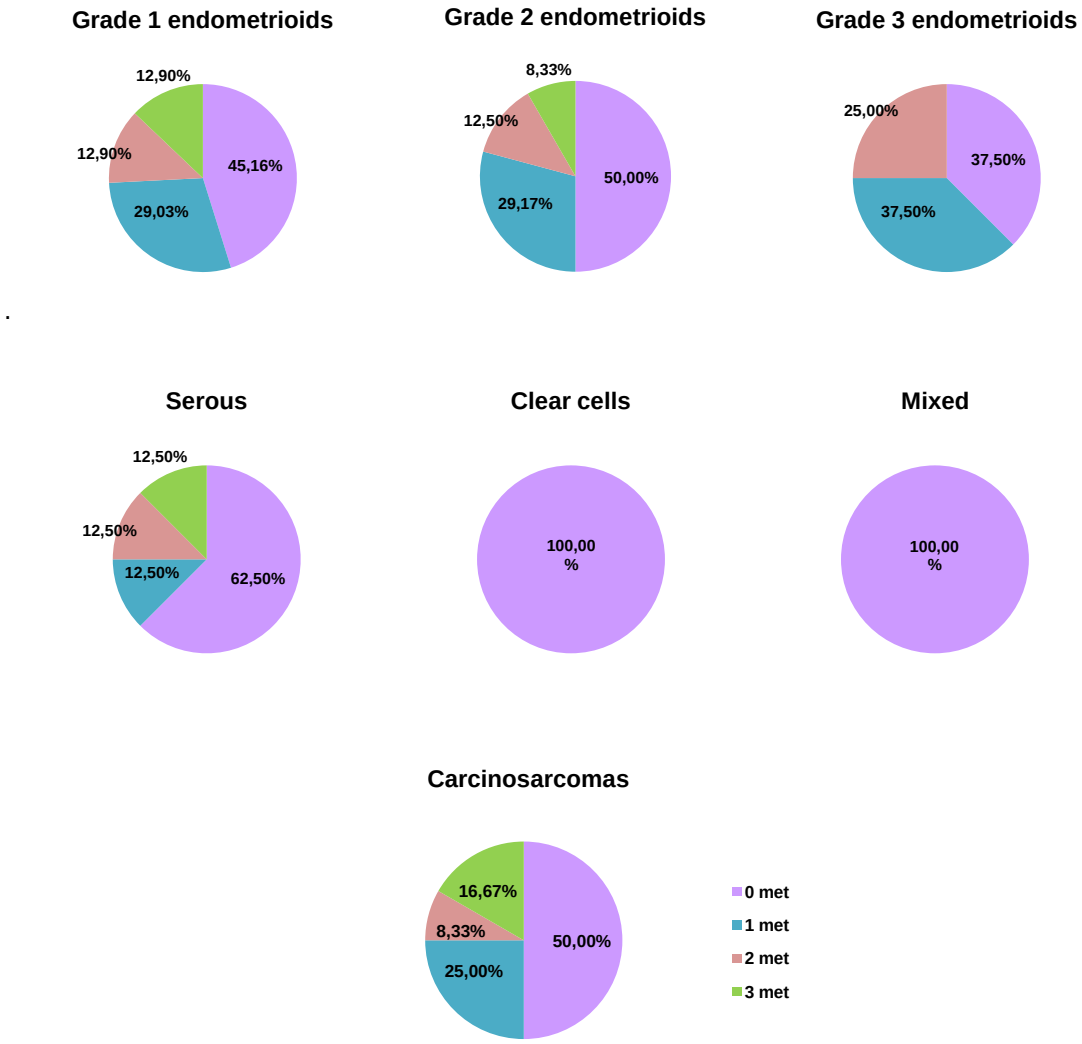

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Figure 20C: Percentage of tumours with 1, 2, 3, or none MMR genes methylated in each type of tumour according to classification by histological subtypes.

However, when we segmented the groups in different histological subtypes, it is been detected that in grade 3 endometrioid there is no presence of cases with three genes methylated and they have the same percentage of patients without methylation than with one of the genes methylated (37.50%). It is the only group which shows a different pattern with the exception of clear cells and mixed carcinomas because they only had one and two sample respectively.

Of note that serous carcinomas was the group with a higher absence of methylation in three genes (62.50%) and carcinosarcomas, the group with more cases with three genes methylated (16.67%).

4. ANALYSIS OF HDAC1, HDAC2 AND HDAC3 PROTEIN EXPRESSION

We carried out the analysis of HDAC1, HDAC2, and HDAC3 protein expression in 86 tumours and 4 cell lines.

The expression of the three proteins was homogeneous in the four cell lines despite of AN3CA and SKUT1 cell lines had a heterozygous mutation p.K9fsX22 in the microsatellite region A_9 of HDAC2 gene[223].

The results in each case and each HDAC are showed in table 24.

Table 24: Results of the analysis of the HDAC1, HDAC2, and HDAC3 protein expression in the 86 endometrial carcinomas included in our study.

Case	HDAC1	HDAC2	HDAC3	Case	HDAC1	HDAC2	HDAC3
1	No	No	No	**44**	Yes	Yes	Yes
2	---	---	---	**45**	Yes	Yes	Yes
3	---	---	---	**46**	Yes	Yes	Yes
4	Yes	Yes	Yes	**47**	No	No	Yes
5	Yes	Yes	Yes	**48**	Yes	Yes	Yes
6	---	---	---	**49**	No	No	Yes
7	Yes	Yes	Yes	**50**	No	No	Yes
8	Yes	No	No	**51**	Yes	Yes	Yes
9	Yes	Yes	Yes	**52**	Yes	No	Yes
10	Yes	Yes	Yes	**53**	Yes	Yes	Yes
11	No	Yes	No	**54**	Yes	No	Yes
12	No	Yes	No	**55**	Yes	Yes	Yes
13	---	---	---	**56**	Yes	Yes	Yes
14	No	Yes	No	**57**	Yes	No	Yes
15	Yes	Yes	Yes	**58**	Yes	No	Yes
16	Yes	Yes	Yes	**59**	Yes	Yes	Yes
17	Yes	No	No	**60**	Yes	No	Yes
18	No	No	No	**61**	Yes	Yes	Yes
19	No	Yes	No	**62**	Yes	Yes	Yes
20	Yes	Yes	Yes	**63**	No	No	Yes
21	Yes	Yes	Yes	**64**	Yes	Yes	Yes
22	Yes	Yes	Yes	**65**	Yes	No	Yes
23	Yes	Yes	Yes	**66**	Yes	Yes	Yes
24	Yes	Yes	Yes	**67**	Yes	Yes	Yes
25	No	Yes	No	**68**	Yes	Yes	Yes
26	Yes	Yes	Yes	**69**	Yes	Yes	Yes
27	Yes	Yes	Yes	**70**	Yes	No	Yes
28	Yes	Yes	Yes	**71**	Yes	No	Yes
29	Yes	Yes	Yes	**72**	Yes	Yes	Yes
30	Yes	No	No	**73**	Yes	No	Yes
31	No	No	Yes	**74**	Yes	Yes	Yes
32	Yes	Yes	Yes	**75**	Yes	Yes	Yes
33	Yes	Yes	Yes	**76**	Yes	Yes	Yes
34	No	No	No	**77**	Yes	Yes	Yes
35	Yes	Yes	Yes	**78**	Yes	Yes	Yes
36	Yes	Yes	Yes	**79**	Yes	Yes	Yes
37	No	Yes	Yes	**80**	Yes	No	Yes
38	Yes	No	Yes	**81**	No	No	Yes
39	Yes	Yes	Yes	**82**	Yes	Yes	Yes
40	No	No	Yes	**83**	Yes	Yes	Yes
41	Yes	Yes	Yes	**84**	Yes	No	Yes
42	Yes	Yes	Yes	**85**	Yes	No	No
43	Yes	Yes	Yes	**86**	Yes	No	Yes

We did not have any protein expression either HDACs non β-actina in cases 2, 3, 6, and 13 maybe because of a problem in the extraction or manipulation process.

Of the other 82 cases, 33 (40.24%) showed absence of at least one of the HDACs. 3 tumours (3.66%) showed loss of expression of the three HDACs, 16 (19.51%) loss of expression of two of them and in 13 cases (15.86%) loss of expression of only one HDAC.

Finally, we have to highlight that in 16 tumours (19.51%) there was not expression of HDAC1, in 26 cases (31.71%) there was an absence of HDAC2 and in 12 patients (14.63%), of HDAC3.

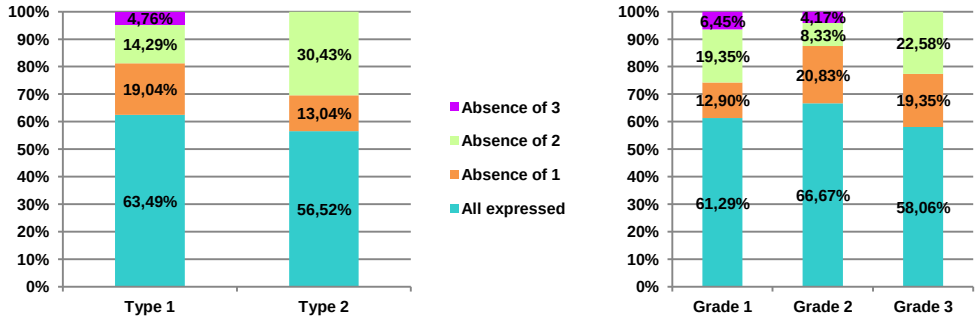
Subdividing by subtypes and grades of tumours we observed the results showed in the table 25:

Table 25: Percentage of tumours with presence or absence of the HDAC1, HDAC2, and HDAC3 protein expression in each type of tumour according to the clinical-pathological classification (type 1 or endometrioids and type 2 or non-endometrioids), histological grade (grade 1, 2, and 3) and histological subtypes (grade 1 endometrioids, grade 2 endometrioids, grade 3 endometrioids, serous, clear cells, mixed and carcinosarcoma). E1: grade 1 endometrioid carcinoma; E2: grade 2 endometrioid carcinoma; E3: grade 3 endometrioid carcinoma; C.C.: clear cells endometrial carcinoma; CR: carcinosarcoma.

Expression / Type	All expressed	Absence of expression	Absence of HDAC1	Absence of HDAC2	Absence of HDAC3
Type 1	40 (63.49%)	23 (36.51%)	10 (15.87%)	20 (31.74%)	8 (12.70%)
Type 2	13 (56.52%)	10 (43.48%)	6 (26.09%)	7 (30.43%)	4 (17.39%)
Grade 1	19 (61.29%)	12 (38.71%)	7 (22.58%)	11 (35.48%)	5 (16.13%)
Grade 2	16 (66.67%)	8 (33.33%)	3 (12.50%)	6 (25.00%)	3 (12.50%)
Grade 3	18 (58.06%)	13 (41.94%)	6 (19.35%)	10 (32.26%)	4 (12.90%)
E1	19 (61.29%)	12 (38.71%)	7 (22.58%)	11 (35.48%)	5 (16.13%)
E2	16 (66.67%)	8 (33.33%)	3 (12.50%)	6 (25.00%)	3 (12.50%)
E3	5 (62.50%)	3 (37.5%)	0 (0.00%)	3 (37.50%)	0 (0.00%)
Serous	4 (50.00%)	4 (50.00%)	3 (37.50%)	2 (25.00%)	2 (25.00%)
C. C.	0 (0.00%)	1 (100.00%)	0 (0.00%)	1 (100.00%)	1 (100.00%)
Mixed	2 (100.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
CR	7 (58.33%)	5 (41.67%)	3 (25.00%)	4 (33.33%)	1 (8.33%)

Every group had in common that more than 50% of cases had expression of all HDACs and HDAC2 was the most absent protein in all of them excepting serous carcinomas.

Attending to the number of HDAC proteins with lack of expression in each type, we observed the followed results (figure 21):



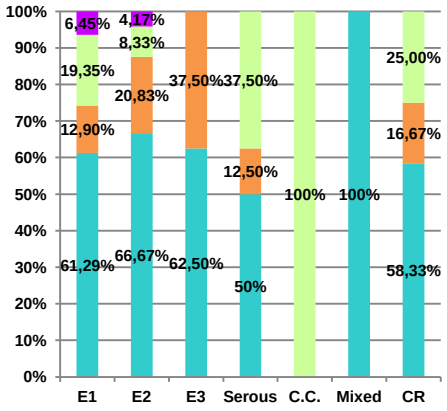


Figure 21: Percentage of tumours with expression of 0, 1, 2, or 3 or HDACs proteins in each type of tumour according to the clinical-pathological classification (type 1 or endometrioids and type 2 or non-endometrioids), histological grade (grade 1, 2, and 3) and histological subtypes (grade 1 endometrioids, grade 2 endometrioids, grade 3 endometrioids, serous, clear cells, mixed and carcinosarcoma). E1: grade 1 endometrioid carcinoma; E2: grade 2 endometrioid carcinoma; E3: grade 3 endometrioid carcinoma; C.C.: clear cells endometrial carcinoma; CR: carcinosarcoma.

The most important difference between the group of endometrioid carcinomas and non-endometrioid carcinomas was the no presence in the second group of tumours with absence of the three HDACs protein expression. This fact was observed when we subdivided by grades in the group of grade 3 carcinomas, which showed a higher number of tumours with non-expression of two HDACs but no cases with the three proteins affected. Attending to a more accurate classification, we detected that grade 3 endometrioid carcinomas was a especial group showing only expression in two (37.50%) or three (62.50%) HDACs, but never the lack of two or three proteins expression. On the other hand, serous carcinomas did not show absence of the three proteins expression but yes the highest percentage of tumours without two HDACs expression (37.50%). Carcinosarcomas showed its own profile without any tumour with absence of the three proteins expression and the only clear cells tumour we had, didn´t present two HDACs expression. The two mixed tumours showed every HDAC expressed.

5. TELOMERE LENGTH STUDY

Telomere length is related to different pathological status included tumorigenesis. It is not clear if it consists in a cause or a consequence of the development of the tumour but it has been associated with several types of tumours included ovarian carcinoma.

The results are showed in supplementary information 5 but here we show the data about the average telomeric length and standard deviations for each group according to the clinical-pathological and histological classification as well as the results of the statistical comparison (table 26).

Table 26: Average telomeric length and standar deviation (STDV) in each type of tumour according to the clinical-pathological classification (type 1 or endometrioids and type 2 or non-endometrioids), histological grade (grade 1, 2, and 3) and histological subtypes (grade 1 endometrioids, grade 2 endometrioids, grade 3 endometrioids, serous, clear cells, mixed and carcinosarcoma). E1: grade 1 endometrioid carcinoma; E2: grade 2 endometrioid carcinoma; E3: grade 3 endometrioid carcinoma; C.C.: clear cells endometrial carcinoma; CR: carcinosarcoma.

Type					Grade					Subtype				
	Number of cases	Average	STDV	p value		Number of cases	Average	STDV	p value		Number of cases	Average	STDV	p value
Type 1	63	1.23	0.61	0.64	G1	31	1.25	0.56	0.84	E1	31	1.25	0.56	0.51
					G2	24	1.31	0.69		E2	24	1.31	0.69	
					G3	31	1.21	0.64		E3	8	0.90	0.47	
Type 2	23	1.32	0.66							Serous	8	1.48	0.67	
										C.C.	1	1.54	---	
										Mixed	2	0.97	0.53	
										CR	12	1.25	0.71	

Grade 3 endometrioid and mixed carcinomas were the groups with lower telomeric length while serous and clear cells showed the highest.

All the distributions were non-parametric so we applied a U Mann Whitney to make the comparison between groups and one-way Anova to compare more than two groups. The program precluded the clear cell tumour group because of having only one case.

When comparing relative length depending on type, grade and subtype groups of tumours we didn´t find any significant result but when we did it two by two groups we observed significant differences always between mixed carcinomas were matched up to other groups (table 27).

Table 27: p-values resulting from the comparision of the telomeric length two by two groups of tumours according. E3: grade 3 endometrioid carcinoma; C.C.: clear cells endometrial carcinoma; CR: carcinosarcoma.

	Type 1	Type 2	G1	G2	G3	E3	Serous	Mix.	CR
Type 1	---	0.64	---	---	---	---	0.35	<0.001	0.99
Type 2	---	---	0.88	0.96	---	0.23	---	---	---
G1	---	---	---	0.84	0.67	0.11	0.41	<0.001	0.95
G2	---	---	---	---	0.63	0.18	0.59	<0.001	0.75
G3	---	---	---	---	---	---	---	---	---
E3	---	---	---	---	---	---	0.13	<0.001	0.37
Serous	---	---	---	---	---	---	---	<0.001	0.56
Mix.	---	---	---	---	---	---	---	---	<0.001
CR	---	---	---	---	---	---	---	---	---

6. *TERT-1327C>T* AND *TERC-63G>A* POLYMORPHISMS STUDY

C and G allele of *TERT-1327C>T* and *TERC-63G>A* polymorphisms respectively are related to a lower telomerase activity[220,221]. The results of genotype studies are showed in supplementary information. In next table are represented the apparition frequencies of each genotype according with tumour type, grade and subtype (table 28).

Table 28: Genotypes distribution of the *TERT-1327C>T* and *TERC-63G>A* polymorphisms and the p-values resulting from the comparison of the groups of tumours according to the clinical-pathological classification (type 1 or endometrioids and type 2 or non-endometrioids), histological grade (grade 1, 2, and 3) and histological subtypes (grade 1 endometrioids, grade 2 endometrioids, grade 3 endometrioids, serous, clear cells, mixed and carcinosarcoma). E1: grade 1 endometrioid carcinoma; E2: grade 2 endometrioid carcinoma; E3: grade 3 endometrioid carcinoma; C.C.: clear cells endometrial carcinoma; CR: carcinosarcoma.

Tumour	*TERT-1327C>T*				*TERC-63G>A*			
	C/C	C/T	T/T	p value	G/G	G/A	A/A	p value
Type 1	19 30.2%	37 58.7%	7 11.1%	0.027	4 6.3%	18 28.6%	41 65.1%	0.739
Type 2	13 56.5%	6 26.1%	4 17.4		1 4.3%	5 21.7%	17 73.9%	
Grade 1	7 22.6%	20 64.5%	4 12.9%	0.013	2 6.5%	9 29.0%	20 64.5%	0.929
Grade 2	8 33.3%	15 62.5%	1 4.2%		1 4.2%	5 20.8%	18 75.0%	
Grade 3	17 54.8%	8 25.8%	6 19.4%		2 6.5%	9 29.0%	20 64.5%	
E1	7 22.6%	20 64.5%	4 12.9%	0.038	2 6.5%	9 29.0%	20 64.5%	0.752
E2	8 33.3%	15 62.5%	1 4.2%		1 4.2%	5 20.8%	18 75.0%	
E3	4 50.0%	2 25.0%	2 25.0%		1 12.5%	4 50.0%	3 37.5%	
Serous	4 50.0%	4 50.0%	0 0.0%		1 12.5%	3 37.5%	4 50.0%	
C. C.	1 100.0%	0 0.0%	0 0.0%		0 0.0%	0 0.0%	1 100.0%	
Mixed	0 0.0%	1 50.0%	1 50.0%		0 0.0%	0 0.0%	2 100.0%	
CR	8 66.7%	1 8.3%	3 25.0%		0 0.0%	2 16.7%	10 83.3%	

We observed that C/T genotype of *TERT-1327C>T* was significantly more frequent in type 1 than type 2 tumours. Moreover, there were significant differences when comparing grades of tumours noticing that C/T genotype decreases with grade of tumour. In *TERC-63G>A* we didn´t find any significant different in the obtained results.

When grouping genotypes containing C allele in *TERT-1327C>T* we didn´t obtained significant differences in any comparison. However, to have T allele is associated with endometrioid tumours (table 29).

Table 29: Distribution of the genotypes grouping of the *TERT-1327C>T* polymorphism and the p-values resulting from the comparision between the type 1 or endometrioids and the type 2 or non-endometrioids, and among grades 1, 2, and 3.

Tumour	*TERT-1327C>T*							
	C/C+C/T	T/T	p value		T/T+C/T	C/C	p value	
Type 1	56 88.9%	7 11.1%	0.440		44 69.8%	19 30.2%	0.025	
Type 2	19 82.6%	4 17.4%			10 43.5%	13 56.5%		
Grade 1	27 87.1%	4 12.9%	0.264		24 77.4%	7 22.6%	0.375	
Grade 2	23 95.8%	1 4.2%		0.094	16 66.7%	8 33.3%		0.375
Grade 3	25 80.6%	6 19.4%			17 54.8%	14 45.2%		

G and A alleles in *TERC-63G>A* polymorphism didn´t show any relation with any type or grade of tumour.

We carried out a comparison two by two groups with the aim of study the possible differences in the apparition frequencies of C and G alleles observing next results (table 30).

Table 30: p-values resulting from the comparision two by two those tumours which had the allele C in the *TERT-1327C>T* polymorphism and those tumours which had the allele G in the *TERC-63G>A* polymorphism. E3: grade 3 endometrioid carcinoma; C.C.: clear cells endometrial carcinoma; CR: carcinosarcoma.

	TERT-1327C>T C/C+C/T vs T/T	Type 1	Type 2	G1	G2	G3	E3	Serous	Mix.	CR
TERC-63G>A G/G+G/A vs A/A	Type 1	---	0.440	---	---	---	---	0.321	0.99	0.195
Type 1	---	Type 2	---	0.646	0.142	---	0.639	---	---	---
Type 2	0.439	---	G1	---	0.264	0.490	0.398	0.284	0.156	0.335
G1	---	0.439	---	G2	---	0.094	0.080	0.557	0.019	0.061
G2	---	0.932	0.404	---	G3	---	---	---	---	---
G2	---	---	1.000	0.404	---	E3	---	0.131	0.490	1.000
E3	---	0.064	0.166	0.053	---	---	Serous	---	0.035	0.125
Serous	0.404	---	0.452	0.186	---	0.614	---	Mix.	---	0.469
Mix.	0.304	---	0.302	0.420	---	0.283	0.197	---	CR	---
CR	0.533	---	0.228	0.571	---	0.035	0.111	0.533	---	

7. MULTIVARIANT ANALYSIS OF THE RESULTS

Next, it is showed the results obtained by contingency tables when comparing the different groups in each variable studied (table 31).

Table 31: p-values resulting from the comparision of frequencies of the different analyzed alterations according to the clinical-pathological classification (type 1 or endometrioids and type 2 or non-endometrioids), histological grade (grade 1, 2, and 3) and histological subtypes (grade 1 endometrioids, grade 2 endometrioids, grade 3 endometrioids, serous, clear cells, mixed and carcinosarcoma). E1: grade 1 endometrioid carcinoma; E2: grade 2 endometrioid carcinoma; E3: grade 3 endometrioid carcinoma; C.C.: clear cells endometrial carcinoma; CR: carcinosarcoma.

Alterations / Groups	*PTEN*	*TP53*	*CDKN2A*	*PPP2R1A*	*ARID1A*	*PIK3CA*	*CTNNB1*	*KRAS*	*BRAF*	*hMLH1*	Met. *hMLH1*	Met. *hMSH2*	Met. *hMSH6*	NE HDAC1	NE HDAC2	NE HDAC3
Type 1 vs Type 2	0.005	0.002	0.543	0.058	0.154	0.076	0.395	0.070	0.543	0.452	0.055	0.746	0.579	0.281	0.908	0.578
G1 vs G2 vs G3	0.222	0.009	0.408	0.125	0.197	0.232	0.181	0.732	0.408	0.673	0.576	0.335	0.954	0.629	0.702	0.908
Subtypes	0.047	0.025	0.130	0.025	0.023	0.281	0.666	0.420	0.938	<0.001	0.428	0.744	0.881	0.465	0.662	0.169
E1 vs E2 vs E3	0.662	0.510	0.030	0.539	0.162	0.128	0.252	0.069	0.592	0.592	0.465	0.252	0.972	0.252	0.662	0.474
E1+E2 vs E3	0.364	0.263	0.008	0.303	0.367	0.207	0.189	0.028	0.701	0.701	0.271	0.189	0.811	0.189	0.708	0.248
G1+G2 vs G3	0.083	0.002	0.180	0.042	0.544	0.810	0.132	0.977	0.450	0.678	0.381	0.290	0.759	0.893	0.897	0.833

On the other hand, we carried out a multivariant analysis using MULTBiplot program in order to observe how the alterations were associated with the different grades and histological subtypes of endometrial carcinoma. For the multivariant analysis with MULTBiplot program It is needed the presence of at least three groups to compare. Because of that, the classification according with clinical-pathological criteria was taken aside from the study (figures 22 y 23).

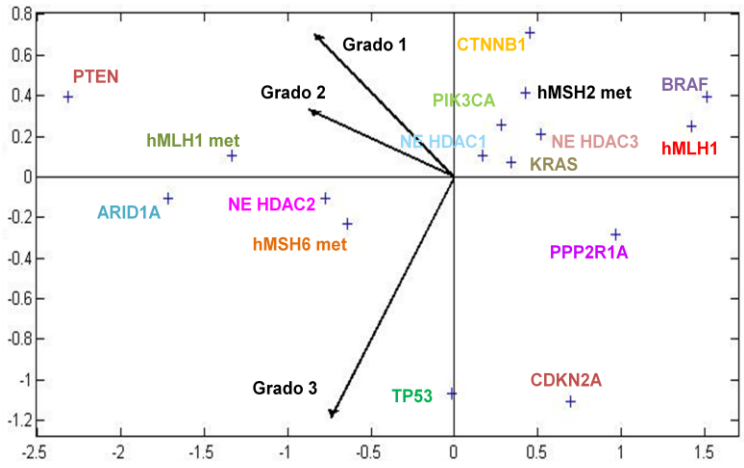


Figure 22: Biplot obtained when comparing the different histological grades.

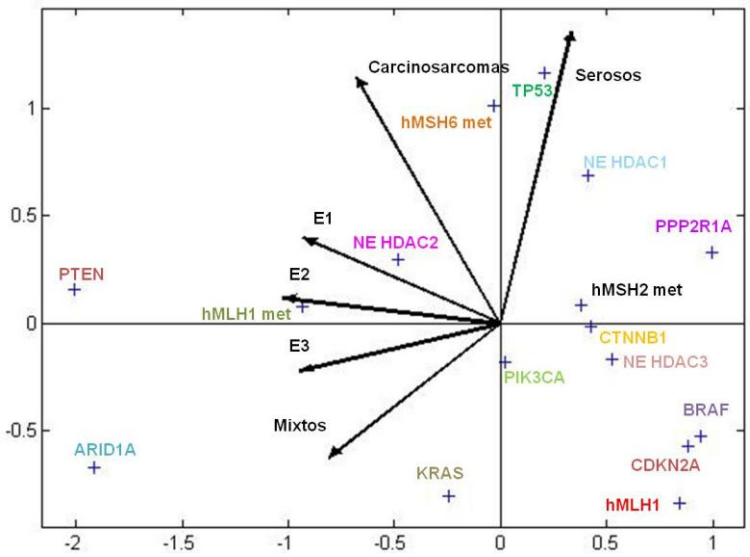


Figure 23: Biplot obtained when comparing the different histological subtypes.

Biplot analysis is usually used as a screening method but we decided to use it to check our results.

The Biplot shows a possible relation between grade 2 tumors and mutations in *PTEN* gene, *hMLH1* gene methylation and an absence of HDAC2 expression whereas grade 3 tumors may be associated with *TP53* mutations. Grade 1 tumours don´t seem to be associated with any alteration. Moreover, grade 1 and grade 2 tend to be closer one each other but both separate from grade 3 due to the different molecular characteristics showed.

On the other hand, grade 1 endometrioid and carcinosarcomas seem to be associated with HDAC2 non-expression, grade 2 endometrioid carcinomas with mutations in *PTEN* gene and *hMLH1* methylation, grade 3 endometriod carcinomas with mutations in *ARID1A*. Moreover, serous carcinomas show a strong association with mutations in *TP53* genes, and slightly lower with *hMSH6* methylation and non-expression of HDAC1.

DISCUSSION

1. STUDY OF NEW GENES IMPLICATED IN THE SPORADIC ENDOMETRIAL CARCINOMA DEVELOPMENT

Sporadic endometrial carcinoma is associated with a hefty number of genetic and epigenetic alterations. As it represents a 95% of all endometrial carcinomas, the study of its molecular characteristics acquires a high interest in order to make a proper diagnosis and find out directed therapies that can combat it.

Currently, many works are looking for new genes implicated in the endometrial carcinoma development. Recent studies, carried out by exomic sequencing, have detected two new targets: *PPP2R1A* y *ARID1A*.

In our work, we have considered opportune a deep analysis of both genes.

1.1 THE *PPP2R1A* GENE

PPP2R1A gene (*protein phosphatase 2, regulatory subunit A, alpha*) codifies for subunit A isoform α of the PP2A complex[117,118]. It is composed by 15 exons that codify 15 HEATs motives acquiring high importance HEATS 5 and 7 because it is the region where are located every pathogenic mutation described up to now in ovarian and uterus cancer[33,125,224]. That two motives and H6 motif are codified by exons 5 and 6 of the *PPP2R1A* gene and currently, they are the only analyzed regions when looking for mutations.

However, in our work we have wanted to analyze all the exons and most adjacent introns of the *PPP2R1A* gene in 31 high-grade carcinomas and only the exons 5 and 6 in the grade 1 and 2 endometrioid carcinomas. We have found three previously described mutations[33,119]: p.S256T (case 29), p.R183Q (case 37), and p.P179R (cases 48, 49 59 and 70). The p.R183Q and p.P179R mutations are located in HEAT5 motif while the p.S256T mutation is located in HEAT7 motif. Of note that samples 37 and 48 were grade 2 and grade 1 endometrioid carcinomas respectively.

It has been described that the distribution of mutations in ovarian cancer is different from that observed in endometrial carcinoma[33,119]; whereas in ovarian cancer around the 70% of mutations in the *PPP2R1A* gene are located in 182 and 183 codons, in the 77% of endometrial carcinomas the codons 179, 256 and 257 are mutated. However, in endometrioid carcinomas it has been detected a higher frequency of mutations in codons 182/183 (similar than in ovarian cancer) than in 256/257 codons. The cause of this different distribution is not clear but could be associated with mutagenesis mechanisms or specific tissue functional effects[224]. In our work, the four serous carcinomas with pathogenic mutations in *PPP2R1A* gene showed codons 179 or 256 altered according with

other authors have described. However, the only mutation found in the codon 183 pertained to a grade 2 endometrioid carcinoma (case 37), although we also observed a change in codon 256 in a grade 1 endometrioid carcinoma (case 48).

Moreover, although every mutation has been described in the exons 5 and 6 of the *PPP2R1A* gene, we have found a probably pathogenic somatic mutation in exon 2 (p.R48Q in the case 52, a grade 3 endometrioid carcinoma) and a non-pathogenic somatic mutation in exon 5 of the gene (p.K194N in the case 45, a grade 2 endometrioid carcinoma) (figure 24).

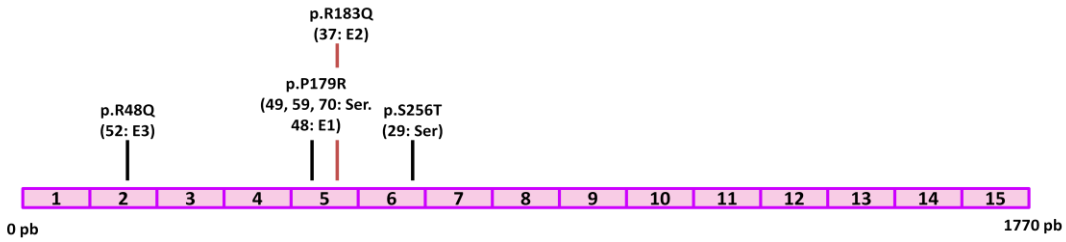


Figure 24: Pathgenic mutations observed in the *PPP2R1A* gene in the study of the 86 endometrial carcinomas. Number and type of carrier tumour are shown. E1: grade 1 endometrioid; E2: grade 2 endometrioid; E3: grade 3 endometrioid; Ser: serous.

On the other hand, in our study we identified, for the first time, a group of mutations in the 5´-UTR region: c.-52A>G (7 cases), c.-73T>C (6 cases), c.-52insC (11 cases), c.-44G>C (1 case), c.-53C>T (1 case), c.-52insCCC (1 case) y c.-53insC (3 cases). The region between c.-53 and c.-60 nucleotides is a sequence with a high number of Citosines and the fact of frequently finding insertions of this nucleotide suggest that is a region susceptible of DNA polymerase sliding (figure 25).

AACGCGAGCTTAGCCCTGGGTAGCGCGGCCAATGGCCGTGGAGCAGCCCCTGTAAACTGGCTCGGGCGCCCCCAC
-164
GCCCGCCCTTCCTTCTTCTCCCAGCATTGCCCCCCCCACGTTTCAGCACAGCGCTGGCCGCAGTCTGACAGGAAAG
-89 -73 -60 -53 -44
GGACGGAGCCAAGATG
-13

Figure 25: Analyzed sequence of the *PPP2R1A* gene in the 5´-UTR región where it can observe the beggining transcription codón (green); the nucleotides where we detected substitutions (purple); the C-rich región between the -53 and the -60 nucleotides (red); and the sequence of the forward nucleotide (5´-3´) (blue) used for the amplification of the analyzed region.

In our work, we observed that in 6 cases the Timine located in -73 position was substituted by a Citosine (cases 9, 20, 38, 39, 59 and 72). In all those cases, the mutation c.-52A>G was present, suggesting a partial linkage disequilibrium because not every tumours with mutation in the -52 position had the change in the -73 position. Moreover, we the miRBASE database showed that the c.-73T>C mutation causes a proper sequence (uggaggagaaggaaggugaug) for the hsa-miR-765 miRNA binding although this result has not been validated by microRNA program.

The TFSEARCH program showed that these changes in the 5´-UTR region caused the appearance or disappearance of transcription factors binding sequences or even a decrease of their binding affinity. The transcription factors affected by these changes are MZF1, SP1, RUNX1/AML-1A and IKAROS, suggesting that these changes do not alter the protein but can cause a deregulation of transcription mechanisms.

The Western blot analysis did not show any variation in the PPP2R1A protein levels independently of the present mutations in the tumours. However, for the first time in our work, we detected the expression of a protein with approximately 110 kDa instead of the 65 kDa expected one and sometimes both protein expressions in the same sample. This phenomenon is not described and we have not found any association with any change in the genomic sequence. Up to now, authors had focused in the analysis of exons 5 and 6 of the gen *PPP2R1A* but not in the expression of the protein. It has been described that the PPP2R1A protein activity is regulated by its cell localization and posttranslational modifications. An acceptable explanation for this new expression pattern observed in our work could be the existence of posttranslational modifications that caused an alteration in the migration pattern of the protein, being necessary more studies to confirm our hypothesis.

Our study showed pathogenic mutations in the *PPP2R1A* gene in 7 of the 86 endometrial carcinomas (8.14%): 4 serous carcinomas and 3 endometrioid carcinomas. This means that the 50% of the serous carcinomas and the 4.76% of the endometrioid (E1: 3.23%, E2: 4.17%, and E3: 12.50%) analyzed in our work, showed mutationes in the *PPP2R1A* gene similar tan the results obtained by other groups[33,36,125].

Many authors have studied the possible association between the presence of mutations in the *TP53* and *PPP2R1A* genes without finding any relation[119,224]. We have observed that the tumours with mutations in the *PPP2R1A* gene carry also mutations in the *TP53* and *PTEN* genes. In the first case, *PPP2R1A* and *TP53* gene mutations do not depend on the type of tumour while *PPP2R1A* and *PTEN* gene correlation mutation is associated with endometrioid carcinomas (table 32):

Table 32: Tumours with pathogenic mutations in the *PPP2R1A* gene and other alterations found in the rest of the studied genes. E1: grade 1 endometrioid carcinoma; E2: grade 2 endometrioid carcinoma; E3: grade 3 endometrioid carcinoma; Ser.: seros endometrial carcinoma.

Cases	Type of tumour	*PPP2R1A*	OTHER ALTERED GENES				
			PTEN	*TP53*	*ARID1A*	*PIK3CA*	*CTNNB1*
29	**Ser**	p.S256T	---	p.R248Q	---	---	---
37	**E2**	p.R183Q	p.R130G	---	---	---	---
48	**E1**	p.P179R	p.R335X	p.G266R	p.Q1614fsX20	---	---
49	**Ser.**	p.P179R	---	---	---	---	p.S37F
52	**E3**	p.R48Q	p.R130Q p.R142W p.R173Y	p.R175C	p.G1549D	p.T1025A p.K1024N	---
59	**Ser.**	p.P179R	---	---	---	---	---
70	**Ser.**	p.P179R	---	p.R175H p.G266R	---	---	---

1.2 THE *ARID1A* GENE

The *ARID1A* gene (*AT-rich interactive domain 1A*) encodes the nuclear protein BAF250a that takes part of the ATP-dependent chromatin remodeling complex: SWI/SNF. The main function of this complex is to mobilise nucleosomes acting as an expression and chromatin dynamic regulator[225].

ARID1A has 20 exons and is mutated in a wide variety of human cancers but mainly in those related to endometrium such as clear cell and endometrioid ovarian carcinomas as well as endometrioid carcinomas of the uterus. In other diseases, it is observed a loss of expression due to the promoter methylation or loss of copies[180,181].

In our work, we have studied all exonic and intronic adjacent regions of the *ARID1A* gene in observing 41 pathogenic mutations without detecting hotspots but observing a trendy of showing more mutations in the biggest exons. Only a mutation was detected in two different tumours: c.5547_5549delG, p.D1850fsX32 (cases 5 and 84) and it had already been observed by other authors in colon and gastric carcinomas[176]. We described it for the first time in endometrial carcinoma. The mutation is located in a G-rich region where it had been described other mutations type insertion (c.5548dupG) in colorrectal, pancreatic and prostate cancers[176]. Similarly, we have found 12 more mutations type insertion/deletion in repetitive sequences. This is a frequent event in the *ARID1A* gene and it has been associated with possible defects in the MMR mechanism[176].

We have also found two pathogenic inframe mutations, one of them already described by other authors (p.Q1334_R1335dupQ)[172,177] and another described for the first time in our work (g.5346_5377del). The Glutamine duplication in the first case (tumor 26) appears in a poly-Q region susceptible to be altered with insertions and deletions of that aminoacid. Its analysis has shown that it does not affect to the protein stability but can change its affinity for the *CDKN1A* gene promoter. Moreover, this Q-rich region could be important in the interaction between the BAF250a complex and other subunits of the SWI/SNF chromatin remodeling complex[172]. On the second place, the g.5346_5377del mutation (case 86) is a loss of 30 nucleotides that causes an aberrant RNA splicing because there is a transcription of the exons 18 and 19 that generates an untimely stop codon that excludes the transcription of the exon 20. Therefore, this mutation affects to NLS domain and causes the loss of the NES domain and three LXXLL domains. LXXLL domains are characteristics of the nuclear receptors coactivators and an alteration in these regions could provoke the impossibility, of the coactivator, to bind itself to the nuclear receptor and the consequent lack of the activation[226] (figure 26).

On the other hand, only two mutations were located in the DNA-binding domain (p.E1019fsX12 y p.N1081fsX11). In the poly-S region we also detected a pathogenic missense mutation described for the first time in our work: p.S1000Y (figure 26).

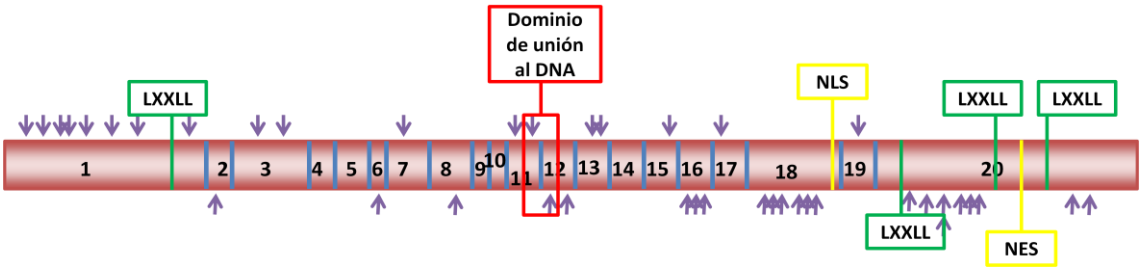


Figure 26: Distribution of the pathogenic mutations observed in the *ARID1A* gene and the localization of the main functional domains. NLS: nuclear licalization signal. NES: nuclear export signal.

Other authors have described the loss of the expression of the BAF250a protein in 39% of the grade 3 endometrioid carcinomas, in 29% of the grade 1 and 2 endometrioid carcinomas, in 18% of the serous carcinomas and in 26% of the clear cell carcinomas. As opposed to other types of tumours, in the endometrial carcinoma, the loss of expression of this protein seems to correlate with the mutations found[21,227].

In our work, when comparing the endometrioid and the non-endometrioid carcinomas we do not find any significance differences as well as when comparing the different grades of tumours. However, when comparing the different histological subtypes we found significance differences due to the presence of mutations in every mixed and clear cell tumours and no serous carcinomas showed any pathogenic mutation in the *ARID1A* gene. This result is similar than other authors have observed in studies with a lower number of samples in which they found a percentage of mutated serous endometrial carcinomas between 0 and 11%[21,36].

In our work, the mutations in the *ARID1A* gene appear mainly associated with mutations in the *PTEN* (72.97%) and *PIK3CA* (18.92%) genes. Given that only the group of the endometrioid carcinomas, of the 30 tumours with *ARID1A* mutated, 23 (76.67%) showed mutations in the PTEN gene and 6 (20%) in *PIK3CA*. Liang et al. has recently described this coexistence[228]. In their work, the analyzed the genes implicated In the PI3K-AKT-mTOR pathway and the *ARID1A* gene in 222 endometrial carcinomas observing a strong coexistence between mutations in the *ARID1A* gene and the *PTEN* or *PIK3CA* genes. Moreover, the observed that in tumours with mutated *ARID1A* and wild type *PTEN* and *PIK3CA* genes there was a deregulation in the protein and phosphorylation levels of the PI3K-AKT-mTOR components confirming the direct interaction between the BAF250a protein and this pathway[228].

The high frequency of mutations in repetitive sequences of the *ARID1A* gene suggests a possible association with alterations in the MMR genes (table 33).

Table 33: Alterations found in the MMR system and the MSI in endometrial tumours with the ARID1A gene mutated. Tumours with mutations in repetitive sequences are highlithed in blue. E1: grade 1 endometrioid carcinoma; E2: grade 2 endometrioid carcinoma; E3: grade 3 endometrioid carcinoma; C.C.: clear cells endometrial carcinoma; MIX.: Mixed carcinomas; CR: carcinosarcoma; ND: undetermined; Exp.: expression; Mut.: mutation; Met.: methylation; Ampl.: amplification.

Cases	Type of tumour	*hMLH1*				*hMSH2*				*hMSH6*				MSI
		Exp.	Mut.	MLPA	Met.	Exp.	Mut	MLPA	Met.	Exp.	Mut.	MLPA	Met.	
2	E2	ND	NO	NO	+	ND	NO	NO	-	ND	ND	ND	-	ND
3	E1	ND	NO	NO	-	ND	NO	NO	-	ND	ND	ND	-	MSI-H
5 (2)	CR	ND	NO	NO	+	ND	NO	NO	+	ND	ND	ND	+	ND
6	E1	ND	NO	NO	+	ND	NO	NO	+	ND	ND	ND	+	ND
14	E2	ND	NO	NO	-	ND	NO	Ampl.	-	ND	ND	ND	-	ND
17	E2	ND	NO	NO	-	ND	NO	NO	-	ND	ND	ND	+	MSS
21	E1	ND	NO	NO	+	ND	NO	NO	-	ND	ND	ND	-	MSI-H
22	E2	ND	NO	NO	-	ND	NO	NO	-	ND	ND	ND	-	ND
23 (2)	MIX.	ND	NO	NO	-	ND	NO	NO	-	ND	ND	ND	-	MSI-H
26	E2	ND	NO	NO	-	ND	NO	NO	-	ND	ND	ND	-	ND
30	CC	ND	IVS3+5GA	NO	-	ND	NO	NO	-	ND	ND	ND	-	ND
31	E1	ND	NO	NO	-	ND	NO	NO	-	ND	ND	ND	+	ND
35	E1	ND	NO	NO	+	ND	NO	NO	-	ND	ND	ND	+	ND
36	E3	ND	NO	NO	-	ND	NO	NO	-	ND	ND	ND	-	ND
39 (2)	CR	ND	NO	NO	-	ND	NO	NO	-	ND	ND	ND	-	ND
40	E1	ND	NO	NO	-	ND	NO	NO	-	ND	ND	ND	-	MSI-H
41	E1	ND	NO	NO	+	ND	NO	NO	+	ND	ND	ND	+	MSS
44	E2	NO	NO	NO	+	YES	ND	ND	-	YES	ND	ND	-	MSI-H
45	E2	NO	NO	NO	+	YES	ND	ND	+	YES	ND	ND	+	MSI-L
48	E1	NO	NO	NO	+	YES	ND	ND	-	YES	ND	ND	-	ND
52	E3	YES	ND	ND	+	YES	ND	ND	-	YES	ND	ND	-	MSI-L
55	E2	NO	NO	NO	+	YES	ND	ND	-	YES	ND	ND	-	MSI-H
58	CR	YES	ND	ND	-	YES	ND	ND	-	YES	ND	ND	-	ND
60	E2	YES	ND	ND	-	YES	ND	ND	-	YES	ND	ND	-	ND
61	E2	YES	ND	ND	-	YES	ND	ND	-	YES	ND	ND	+	ND
66	MIX.	NO	NO	NO	-	YES	ND	ND	-	YES	ND	ND	-	ND
67	E3	YES	ND	ND	+	YES	ND	ND	-	YES	ND	ND	-	ND
68	E1	YES	ND	ND	+	YES	ND	ND	-	YES	ND	ND	-	MSI-L
69	E2	YES	ND	ND	-	YES	ND	ND	-	YES	ND	ND	+	MSI-L
75	E2	NO	NO	NO	+	YES	ND	ND	-	YES	ND	ND	+	ND
76	E1	YES	ND	ND	-	YES	ND	ND	-	NO	NO	NO	-	ND
78	E2	NO	NO	NO	-	YES	ND	ND	-	YES	ND	ND	-	ND
79 (2)	E1	YES	ND	ND	+	YES	ND	ND	-	YES	ND	ND	-	MSI-H
80	E3	YES	ND	ND	-	YES	ND	ND	-	YES	ND	ND	-	MSI-H
82	E3	NO	NO	NO	+	YES	ND	ND	-	YES	ND	ND	+	ND
84	CR	NO	NO	NO	+	YES	ND	ND	-	YES	ND	ND	+	ND
86	E2	YES	ND	ND	-	YES	ND	ND	-	YES	ND	ND	-	MSS

The study of the microsatellite instability in 15 of the 37 patients with mutations in the *ARID1A* gene showed that 12 tumours (80%) presented MSI suggesting that mutations in *ARID1A* are connected with MSI. Of the 13 cases that showed deletion/insertion mutations in repetitive sequences of the *ARID1A* gene, we could analyze the MSI in 5 of them and all of them showed MSI: 4 MSI-H and 1 MSI-L. For want of a highest number of samples to check these results, we could confirm that those mutations in the *ARID1A* gene can be consequence of alterations in the MMR system. However, there is not a correlation between the different analyzed alterations in the *hMLH1*, *hMSH2* and *hMSH6* genes neither with these types of mutations although nor MSI in general.

2. MOLECULAR PROFILES ASSOCIATED TO THE CLINICAL-PATHOLOGICAL CLASSIFICATION

The clinical-pathological classification is based on the dualistic model proposed by Bookman in 1983 in which endometrial carcinomas were subdivided in two big groups: estrogen-dependent, endometrioid or type 1 endometrial carcinomas and non-estrogen-dependent, non-endometrioid or type 2 endometrial carcinomas[7].

Many studies have analyzed the incidence of the molecular alterations presented in the type 1 and 2 endometrial carcinomas. In our work, the analysis of 43 endometrioid and 23 non-endometrioid carcinomas and 28 blood samples (22 from patients with endometrioid carcinomas and 6 from patients with non-endometrioid carcinomas), has allowed us to determine the incidence of alterations in both types of tumours (table 34).

Table 34: Percentage of the incidence of the alterations studied in our work according to that described by other authors (Described) and to we have observed in our results (In this work). Percentages that differ between that described and that observed are highlighted.

Alteration \ Type		Type 1 o endometrioid		Type 2 o non-endometrioid	
		Described	In this work	Described	In this work
MUTATIONS	*PTEN*	37-61%	60%	5-13%	26%
	TP53	10-25%	13%	50-90%	43%
	CDKN2A	0-2%	2%	2-6%	0%
	CDH1	0%	0%	0%	0%
	PPP2R1A	5-10%	5%	20-40%	17%
	ARID1A	30-40%	48%	15-30%	30%
	PIK3CA	35%	21%	30-35%	9%
	CTNNB1	20-45%	17%	0-5%	9%
	KRAS	15-30%	21%	0-5%	4%
	BRAF	1-4%	2%	1-4%	0%
	EGFR	0%	0%	0%	0%
	hMLH1	0-4%	2%	---	4%
	hMSH2	0-4%	0%	---	0%
	hMSH6	0-1%	0%	---	0%
MSI		20-40%	68%	0-5%	67%
METHYLATION	*hMLH1*	45%	44%	---	22%
	hMSH2	---	16%	---	13%
	hMSH6	---	19%	---	35%
LOSS OF EXPRESSION	HDAC1	---	16%	---	26%
	HDAC2	---	32%	---	30%
	HDAC3	---	13%	---	17%

In our work, the *PTEN* gene was mutated in a percentage of non-endometrioid tumours higher than the reported until now by other authors[16,23,54-60]. Likewise, the *TP53* gene showed a percentage of mutated non-endometrioid carcinomas lower than described[25,26,98-100]. In both cases, when comparing the endometrioid and non-endometroid groups, the differences are statistically significant (table 31), which means that there is a clear association between mutations in the *PTEN* and *TP53* and the endometrioid and non-endometrioid carcinomas respectively.

On the other hand, in our work the *BRAF* and *CDKN2A* genes did not appear mutated in the non-endometrioid carcinomas unlike other series[110,112,113,229-232]. In the case of the *BRAF* gene, this discrepancy can be due to the characteristics of the studied population because it seems to be differences in the incidence of mutations depending of the ethnicity of the patients[233]. Respecting to the *CDKN2A* gene, the number of observed mutations by other authors in the endometrial carcinoma is no very high[110,112,113] and it has been described other processes frequently associated to the loss of the expression of the p16 protein such as homozygous deletions of the gene or the hypermethylation of its promoter[110,112,114]. In fact, mutations in the *CDKN2A* gene are no included in many of the gene "panels" suggested for the characterization of the endometrioid and non-endometrioid carcinomas[36].

In the *EGFR* gene we did not observe any mutation. This data confirms what reported by other authors who have found a los number of mutations despite of existing a strong overexpression of the EGFR protein, mainly in non-endometrioid carcinomas (46%)[31].

The percentage of endometrioid and non-endometrioid tumours with the *PIK3CA* gene mutated was lower than described up to now. A plausible cause could be that the CSGE-heteroduplex technique did not detect every mutation present in the analyzed exons. Most of the current studies use techniques such as next generation sequencing that allows detecting all kind of mutations. Furthermore, recent studies have detected a high frequency of mutations in the exons 1 to 7 of the *PIK3CA* gene[57], therefore, it is possible that there are mutations in other exons not included in our study.

The lower frequency of mutations found in the *CTNBB1* gene could be a consequence of the existence of redundant mutations in the *KRAS* gene. Recent studies have reported that in the endometrioid carcinomas the alterations in the *CTNNB1*, *KRAS,* and *SOX17* genes are redundant and are three independent mechanisms of the WNT pathway activation[234-237]. Mutations in any of the three genes would overactive it.

Our study shows a high number of endometrial tumours with MSI (67-68%), percentages pretty higher than described in other works, especially in non-endometrioid carcinomas[20,143,144]. They approaches to the frequency observed in the endometrial carcinoma associated with Lynch syndrome (75%)[142] although in Lynch syndrome the most instable marker usually is BAT26[238] and in our work was D17S250. There are possible factors that can explain these differences. On the one hand, most of the works use markers different from those proposed by Bethesda[141] and the number of markers is smaller than ours[145]. On the other hand, it has to be in mind that our study has a limited number of samples, especially in the case of non-endometrioid tumours.

In Lynch syndrome is very frequent the appearance of mutations in the *hMSH6* (73%), *hMSH2* (29%), and *hMLH1* (31%) genes as a main cause of the MSI[138]. However, in

sporadic endometrial carcinoma the values are much lower[136,141,222], like we have found, and the most frequent alteration in the MMR is the methylation of the *hMLH1* promoter[145,147,150] as a secondary event to other alterations.

Our results showed the *hMLH1* gene methylated in a percentage similar than described[145-149] unlike the *hMSH2* and *hMSH6* genes in which it was observed a percentage smaller (table 23). However, we have not found any correlation between MSI and the methylation of MMR genes promoters. Even the analyzed of gross alterations in these genes did not explain the MSI and was not in concordance with the loss of expression observed by the immunochemical studies. The study of gross alterations was performed for the first time in our work and the results were similar than reported by other authors for the Lynch syndrome (1-20%)[239-241].

We have also studied the expression of the HDAC1, HDAC2 and HDAC3 proteins in the different types of sporadic endometrial carcinomas. Some authors have described a direct relation between the hormone exposure and the increase of HDAC1 and HDAC2 proteins expression[242,243]. A treatment with steroid hormones, such as estrogens and progesterone, induces the overexpression of both proteins in the sound endometrial stroma. Bearing in mind that the endometrioid carcinomas are associated with a previous stimulation with both hormones, it would make sense the overexpression of both proteins in this group of tumours.

We observed that the HDAC2 protein is the histone deacetylase with an absent expression in a higher percentage of endometrial carcinomas. In the non-endometrioid studied here, the HDAC1 protein also loses the expression in a percentage similar to that observed in the HDAC2 protein (table 25). This may be related to increased aggressiveness and worse prognosis that non-endometrioid carcinomas show because the HDAC1 protein has been associated with a worse prognosis and decreased survival of the patients[167,168,244].

In conclusion, according to our results, the endometrioid carcinomas are associated with mutations in the *PTEN* and *ARID1A* genes, with MSI, with *hMLH1* gene methylation, and with an absence of the HDAC2 protein expression whereas the non-endometrioid carcinomas are related to mutations in the *TP53* gene, MSI, and *hMSH6* methylation (figure 27).

To distinguish the endometrioid carcinomas from the non-endometrioid carcinomas, we suggest the analysis of the mutations mainly in the *PTEN* and *TP53* genes but also in the *PPP2R1A*, *KRAS,* and *PIK3CA* genes as well as the study of the methylation of the *hMLH1* promoter as an epigenetic event to bear in mind.

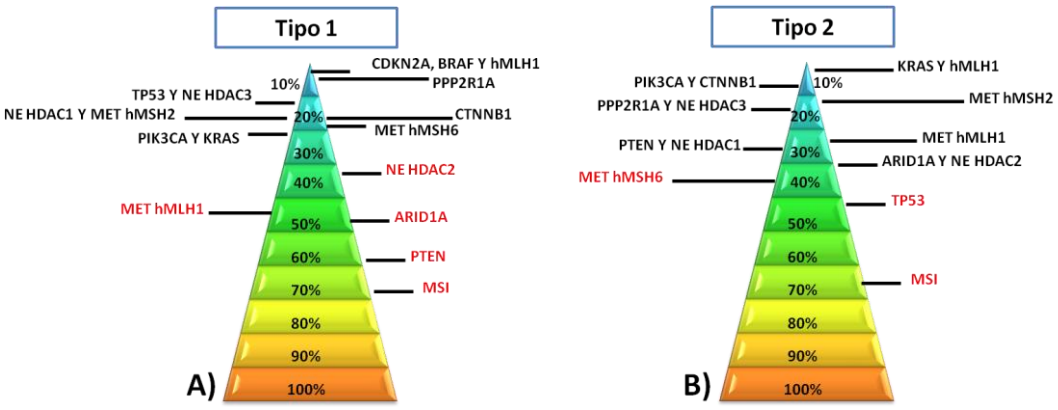


Figure 27: Frequence of appearance of the different alterations analyzed in our work according to the clinical-pathological classification. A) type 1 or endometrioid carcinomas and B) type 2 or non-endometrioid carcinomas. NE: non-expression. MET: methylation.

3. MOLECULAR PROFILES ASSOCIATED TO CLASSIFICATION ACCORDING TO THE HISTOLOGICAL GRADES

The classification of the endometrial carcinomas depending on the clinical-pathological characteristics has been accepted during lots of years as a unique categorization criterion. However, the molecular studies bring up the necessity of looking for other criteria that allow to establish more accurate and clearly defined classifications.

The theory drawn up by Vogelstein, and rounded off by others authors later, suggest that the tumorigénesis would be due to an accumulation of genetic and epigenetic alterations[26,35]. Several authors showed later that grade 3 endometrioid carcinomas present molecular and clinical characteristics similar than the serous endometrial carcinomas[245-247]. Because of that, the grade 3 endometrioid carcinomas started being included in the group of the high-grade carcinomas mainly consisting of the serous and clear cells carcinomas.

In our work, we have studied the genetic and epigenetic characteristics of the endometrial carcinoma according to their histological grades. Following the IFGO and OMS criteria, the tumours have been divided in three groups[15]: grade 1 (grade 1 endometrioid carcinomas), grade 2 (grade 2 endometrioid carcinomas) and grade 3 (grade 3 endometrioid carcinomas, serous carcinomas, clear cell carcinomas, mixed carcinomas carcinomas and carcinosarcomas).

We have analyzed 31 grade 1 (plus 10 blood samples), 24 grade 2 (plus 10 blood samples) and 31 grade 3 tumours (plus 8 blood samples).

In our series, we have compared the classification by histological grades with the clinical-histological classification already described in this work according to the molecular alterations (table 35).

Table 35: Percentage of incidence of the alterations studied in our work when dividing tumours by clinical-pathological characteristics and histological grades.

Alteration \ Criteria		Clinical-pathological		Histological		
		Endometrioid or type 1	Non-endometrioid or type 2	Grade 1	Grade 2	Grade 3
MUTATIONS	*PTEN*	60%	26%	58%	58%	39%
	TP53	13%	43%	10%	13%	39%
	CDKN2A	2%	0%	0%	0%	3%
	CDH1	0%	0%	0%	0%	0%
	PPP2R1A	5%	17%	3%	4%	16%
	ARID1A	48%	30%	35%	59%	39%
	PIK3CA	21%	9%	26%	8%	16%
	CTNNB1	17%	9%	26%	13%	6%
	KRAS	21%	4%	13%	21%	16%
	BRAF	2%	0%	3%	0%	0%
	EGFR	0%	0%	0%	0%	0%
	hMLH1	2%	4%	3%	0%	3%
	hMSH2	0%	0%	0%	0%	0%
	hMSH6	0%	0%	0%	0%	0%
MSI		68%	67%	70%	60%	75%
METHYLATION	*hMLH1*	44%	22%	35%	50%	32%
	hMSH2	16%	13%	16%	21%	10%
	hMSH6	19%	35%	29%	26%	32%
LOSS OF EXPRESSION	HDAC1	16%	26%	23%	13%	19%
	HDAC2	32%	30%	35%	25%	32%
	HDAC3	13%	17%	16%	13%	13%

We observed many similarities with the results obtained when we divided the tumours according with the clinical-pathological characteristics.

First, the classification by histological grades showed that grade 1 and 2 carcinomas have very similar molecular characteristics. We only found differences in the incidence of mutations of the genes *ARID1A, PIK3CA,* and *CTNNB1* and in the methylation of the *hMLH1* gene. At the same time, these characteristics shared a high similarity with those observed in the endometrioid or type 1 carcinomas.

On the other hand, the grade 3 carcinomas showed molecular features different than grade 1 and 2 carcinomas but similar than those observed in type 2 or non-endometrioid carcinomas. These results were confirmed by a multivariate analysis with the MULTBiplot program in which it has detected a high proximity between the axis that represented the grade 1 and grade 2 carcinomas and a big remoteness between that two axis and the axis of the grade 3 endometrial carcinomas (figures 22 and 23).

Similarly than occurred when we compared the type 1 or endometrioid with type 2 or non-endometrioid carcinomas, the low-grade carcinomas (G1+G2) showed differences with high-grade carcinomas (G3) in the percentages of tumours with mutations in the *PTEN*, *PPP2R1A,* and *TP53* genes.

The grade 3 endometrioid carcinomas is an histological subtype that shows molecular characteristics halfway between type 1 and type 2 carcinomas (figure 28).

Therefore, our results show that the classification of endometrial carcinomas according to the histological grade presents a high similarity with the clinical-pathological classification and does not contributing improvements in the molecular classification of the endometrial tumours.

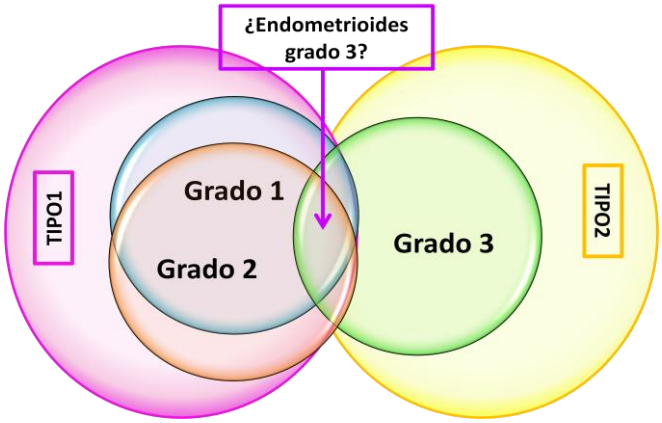


Figure 28: Graphic that shows the relation among the grade 1, 2, and 3 endometrial carcinomas according to the molecular characteristics observed in our work. In the intersection region of the three groups, it would be located the grade 3 endometrioid carcinomas because they show characteristics halfway of the three grades. Grade 1 and grade 2 endometrial carcinomas show characteristics almost identic to type 1 or endometrioid carcinomas whereas the grade 3 endometrial carcinomas could be considerated thegroup of the type 2 or non-endometrioid carcinomas.

This classification has not been used in any molecular study due to there has been a direct evolution from the clinical-pathological classification to the classification by histological subtypes. Some authors like McConechy et al.[36] have proposed that grade 3 endometrioid have to be considered in a separate group because, although they show similarities with grade 1 and grade 2 carcinomas, the differ in some aspects and present a higher incidence of mutations in the *TP53* gene.

In our study, we checked how the classifications previously mentioned are not adequate when establishing molecular profiles that can be useful for future clinical and therapeutic advances. According to our results, we considered more adequate the classification of the endometrial carcinomas according to the histological subtypes.

4. MOLECULAR PROFILES ASSOCIATED TO CLASSIFICATION ACCORDING TO THE HISTOLOGICAL SUBTYPES

4.1 LOW-GRADE ENDOMETRIOID CARCINOMAS

In our work, we have studied 55 low-grade endometrioid carcinomas: 31 grade 1 and 24 grade 2. Both types showed a high frequent of mutations in the *PTEN* gene whereas the *PIK3CA* gene was mutated most frequently in the grade 1 endometrioid carcinomas (table 19). The coexistence of both genes in a 12.73% of cases confirms the findings of the other authors[16,23,54-60] and has been attributed to the participation of the *PTEN* gene in other cell signaling pathways responsible, for example, of the maintenance of the genomic integrity[248].

Cases 45 and 79 showed two alterations with a triple peak pattern in the sequences of the exons 7 and 8 respectively. By the techniques used in our study was not possible to clarify the type of alteration present in either case but they could be chromosomal translocations because they are very frequent events in endometrial carcinoma mostly affecting to genes involved in the PI3K-AKT-mTOR, WNT, EGFR-RAS-MAPK, retinoblastoma, and apoptosis signaling pathways[234].

It would be convenient to study these samples by other kind of techniques that can detect these aberrations, such as FISH, because if there was a translocation it could be affected other genes located in the chromosome 10. When analyzing the rest of the alterations presented in these tumours, it was observed a high genetic and epigenetic instability (table 36)

Table 36: Genetic and epigenetic alterations presented in the cases 45 and 79, both of them are low-grade endometrioid tumours Exp.: expression; Met: methylation; MSI: microsatellite instability; E1: grade 1 endometrioid carcinomas; E2: grade 2 endometrioid carcinomas.

Cases	Type of tumour	ALTERATIONS SHOWED								
		Mutation in *PTEN*	Mutation in *TP53*	Mutation in *ARID1A*	Mutation in *KRAS*	Exp. hMLH1	Met. *hMLH1*	Met. *hMSH2*	Met. *hMSH6*	MSI
45	E2	Traslocation?	---	p.Q546fsX72 p.R1528X	p.G12D	NO	+	+	+	MSI-L
79	E1	Traslocation?	p.C135Y	p.P1175fsX4/ p.E1864fsX35	---	YES	+	-	-	MSI-H

MSI was also a frequent event in the low-grade endometrioid studied in our work (table 37). We could analyze it in 10 patients with grade 1 and 10 patients with grade 2 endometrioid carcinoma. Of the 20 samples analyzed, 13 cases (65%) showed MSI, which is higher than that observed by other authors in sporadic endometrial carcinomas (20-40%)[20,143,144] but similar to that described in endometrial carcinoma associated with Lynch syndrome[142]. As mentioned above, these differences may be due to the difference in number and type of markers analyzed in other series or the small number of samples

studied in our work. As noted in other studies, mutations, methylation and gross rearrangements in MMR genes did not explain the frequent MSI and the lack of protein expression (detected by immunohistochemical techniques) present in low-grade endometrioid carcinomas (table 37). However, it was frequent the promoter methylation of the *hMLH1* gene (35% of cases) regardless of the presence or absence of MSI.

Methylation of the *hMLH1* gene has been described in a percentage of endometrioid carcinomas similar to that detected by us (45%)[145-149]. It occurs since early stages of the tumour development because it has been observed in atypical (33%) and endometrial (3%) hyperplasias[146]. In our work the percentage of cases with *hMLH1* methylated increased with the grade of the tumour being already common in the grade 1 endometrioid (35% of cases). However, methylation in *hMLH1* is proposed as a secondary event so other alterations are needed in order to, coupled to methylation, lead an inactivation of the protein[55,145,146,148,149].

Table 37: Low-grade endometrioid carcinomas that showed alterations in some of the MMR factors. The immunohistochemistry was carried out from the case 42. It was studied the promoter methylation in the *hMLH1*, *hMSH2,* and *hMSH6* genes in every tumour. MLPA assays and the mutational analysis were carried out from case 1 to 41 and from case 42 only in those cases with negative immunohistochemistry. The study of the *hMSH6* gene was only carried out in the case 76. Exp.: expression; Mut.: mutation; Met.: methylated; ND: undetermined; Ampl.: amplification; E1: grade 1 endometrioid carcinoma; E2: grade 2 endometrioid carcinoma.

Cases	Type of tumour	hMLH1				hMSH2				hMSH6				MSI
		Exp.	Mut.	MLPA	Met.	Exp.	Mut.	MLPA	Met.	Exp.	Mut.	MLPA	Met.	
2	**E2**	ND	NO	NO	+	ND	NO	NO	-	ND	ND	ND	-	ND
3	**E1**	ND	NO	NO	-	ND	NO	NO	-	ND	ND	ND	-	MSI-H
4	**E2**	ND	NO	NO	+	ND	NO	NO	++	ND	ND	ND	-	ND
6	**E1**	ND	NO	NO	+	ND	NO	NO	++	ND	ND	ND	++	ND
7	**E1**	ND	NO	NO	++	ND	NO	NO	++	ND	ND	ND	++	ND
8	**E1**	ND	YES	Ampl.	++	ND	NO	NO	-	ND	ND	ND	-	ND
12	**E1**	ND	NO	NO	++	ND	NO	NO	+	ND	ND	ND	-	ND
13	**E2**	ND	NO	NO	++	ND	NO	NO	++	ND	ND	ND	++	ND
14	**E2**	ND	NO	NO	-	ND	NO	Ampl.	-	ND	ND	ND	-	ND
17	**E2**	ND	NO	NO	-	ND	NO	NO	-	ND	ND	ND	+	MSS
21	**E1**	ND	NO	NO	+	ND	NO	NO	-	ND	ND	ND	-	MSI-H
31	**E1**	ND	NO	NO	-	ND	NO	NO	-	ND	ND	ND	+	ND
34	**E1**	ND	NO	NO	+	ND	NO	NO	+	ND	ND	ND	-	ND
35	**E1**	ND	NO	NO	+	ND	NO	NO	-	ND	ND	ND	+	ND
37	**E2**	ND	NO	NO	+	ND	NO	NO	-	ND	ND	ND	-	MSI-H
40	**E1**	ND	NO	NO	-	ND	NO	NO	-	ND	ND	ND	-	MSI-H
41	**E1**	ND	NO	NO	+	ND	NO	NO	+	ND	ND	ND	+	MSS
42	**E1**	YES	ND	ND	+	YES	ND	ND	+	YES	ND	ND	+	MSS
43	**E1**	YES	ND	ND	-	YES	ND	ND	-	YES	ND	ND	-	MSI-L
44	**E2**	NO	NO	NO	++	YES	ND	ND	-	YES	ND	ND	-	MSI-H
45	**E2**	NO	NO	NO	-	YES	ND	ND	+	YES	ND	ND	+	MSI-L
50	**E1**	YES	ND	ND	-	YES	ND	ND	-	YES	ND	ND	-	MSI-L
54	**E2**	YES	ND	ND	+	YES	ND	ND	-	YES	ND	ND	+	MSI-H
55	**E2**	NO	NO	NO	++	YES	ND	ND	-	YES	ND	ND	-	MSI-H
57	**E2**	YES	ND	ND	+	YES	ND	ND	++	YES	ND	ND	-	ND
61	**E2**	YES	ND	ND	-	YES	ND	ND	-	YES	ND	ND	+	ND
64	**E1**	YES	ND	ND	-	YES	ND	ND	-	YES	ND	ND	+	ND
68	**E1**	YES	ND	ND	++	YES	ND	ND	-	YES	ND	ND	-	MSI-L
69	**E2**	YES	ND	ND	-	YES	ND	ND	-	YES	ND	ND	+	MSI-L
71	**E1**	NO	NO	NO	-	YES	ND	ND	-	YES	ND	ND	-	ND
73	**E1**	YES	ND	ND	-	YES	ND	ND	-	YES	ND	ND	+	ND
75	**E2**	NO	NO	NO	+	YES	ND	ND	-	YES	ND	ND	+	ND
76	**E1**	YES	ND	ND	-	YES	ND	ND	-	NO	NO	NO	-	ND

Cases	Type of tumour	hMLH1				hMSH2				hMSH6				MSI
		Exp.	Mut.	MLPA	Met.	Exp.	Mut.	MLPA	Met.	Exp.	Mut.	MLPA	Met.	
77	**E1**	YES	ND	ND	+	YES	ND	ND	-	YES	ND	ND	-	ND
78	**E2**	NO	NO	NO	-	YES	ND	ND	-	YES	ND	ND	-	ND
79	**E1**	YES	ND	ND	+	YES	ND	ND	-	YES	ND	ND	-	MSI-H

On the other hand, our study reflects a fact not described by other authors yet but previously noted in our laboratory[222]: in the endometrioid carcinomas, mutations in the *PTEN* gene frequently coincide with methylation in the MMR genes. 59.38% of tumours with *PTEN* mutated in our work had, at least, one of the MMR genes methylated and mostly, the *hMLH1* gene. Given that the methylation of the MMR genes, and particularly of the *hMLH1*, is an early event in the tumorigenesis[146], the malfunction cannot correct the mistakes produced during the replication. This affects the genes involved in the tumour development and, as observed in our study, especially *PTEN*. However, when there is a methylation of the MMR genes, the mutations detected in the *PTEN* gene are neither insertion/delection type nor they are located in repetitive sequences (table 5, supplementary information 6). This suggests that the methylation of the MMR genes does not directly affect the occurrence of mutations in the *PTEN* gene but contributes to the disruption of important genes involved in other repairing mechanisms. For instance, it has been described mutations situated in repetitive sequences in the BRCA1 gene when there is MSI and alterations in the MMR system. These mutations, due to a defective MMR mechanism, may affect the proper functioning of the BRCA1 protein and hence the repairing of errors in other genes involved in the tumor development[249].

Moreover, it has been described the frequent occurrence of mutations in the *KRAS* gene in early stages of the tumor development process, being altered in the atypical endometrial hyperplasias[250-252]. Other studies have detected *KRAS* mutated in approximately 14% of the sporadic endometrial carcinomas existing differences depending on the subtypes[230]. In our case, 14% of the low-grade endometrioid carcinomas showed mutations in the *KRAS* gene. All were missense being the most frequent c.G35A (p.G12D). The percentage of tumours with *KRAS* mutated was lower in the grade 1 than in the grade 2 endometrioid carcinomas (table 19), which supports theories than affirm that the progression of the endometrioid tumours towards a higher grade is accompanied by mutations in the *KRAS* genes among others[26].

Of the 9 low grade endometrioid carcinomas that showed *KRAS* mutated, we could study the MSI in 5 patients (cases 15, 45, 54, 65, and 68) and analyzed the presence of mutations in other genes related to the development of the sporadic endometrial carcinoma (table 38).

Table 38: Genetic and epigenetic alterations presented in those low-grade endometrioid tumours that showed pathogenic mutations in the *KRAS* gene. Exp.: expression; Met: methylation; ND: undetermined; MSI: microsatellite instability; E1: grade 1 endometrioid carcinomas; E2: grade 2 endometrioid carcinomas.

Cases	Type of tumour	*KRAS*	*PTEN*	MSI	OTHER FACTORS							
					ARID1A	*CTNNB1*	Met. *hMLH1*	Met. *hMSH2*	Met. *hMSH6*	Exp. HDAC1	Exp. HDAC2	Exp. HDAC3
15	**E2**	p.G12V	---	MSS	---		-	-	-	YES	YES	YES
28	**E2**	p.G12V	---	ND	---		-	-	-	YES	YES	YES
34	**E1**	p.G12D	---	ND	---	p.G34R	+	+	-	No	No	No
35	**E1**	p.G13D	p.R233X (H)	ND	p.Q758fsX75	---	+	-	+	YES	YES	YES
45	**E2**	p.G12D	Traslocation?	MSI-L	p.Q546fsX72; p.R1528X	---	-	+	+	YES	YES	YES
54	**E2**	p.G12D	p.N323fsX1	MSI-H	---	---	+	-	+	YES	No	YES
60	**E2**	p.G12D	p.R130G/ p.R130L	ND	p.R1446X	---	-	-	-	YES	No	YES
65	**E1**	p.G12V	IVS6+1G>A	MSS	---	---	-	-	-	YES	No	YES
68	**E1**	p.G13D	---	MSI-L	p.T1438fsX6	p.S37F; p.G34R	+	-	-	YES	YES	YES

Our results show that in low-grade endometrioid carcinomas mutations in *KRAS* gene are not always accompanied by mutations in the *PTEN* gene and MSI and therefore, they may be an event earlier in the tumour development process. This supports some recent studies in which it has been reported that only 50% of the studied tumours with mutations in *KRAS* show the *PTEN* gene mutated; 14%, MSI-H; and other 14%, MSS[253]. However, it refutes the proponed theories that support that mutations in the KRAS genes occur in early stages of the tumorigénesis but they are never a trigger event. Those cases, in which there is a coexistence of mutations in the *PTEN* and *KRAS* genes[250-252], could be treated by combined therapies that act upon both affected pathways.

We did not detect any tumour with concomitant mutations in the *KRAS* and *BRAF* genes. Other authors support that mutations in both genes are exclusive[254]. To complete the analysis of the RAS-RAF-MEK-ERK, pathway, it would be convenient the study of other factors which alterations have been associated with the development of the endometrioid carcinomas such as RASSF1A, RASSF2A, hDAB2IP, BLU, SPROUTY-2, and RSK4 proteins[255-257].

Mutations in the *CTNNB1* gene, as well as in the *KRAS* gene, have been observed in early stages of the tumorigénesis and are involved in the acquisition of a higher tumour grade[258,259]. In our work, we have detected that 18% of the low-grade endometrioid carcinomas present mutations in the *CTNNB1* gene, similar to that observed in other studies[36] (table 39).

Table 39: Genetic and epigenetic alterations presented in those low-grade endometrioid tumours that showed pathogenic mutations in the *CTNNB1* gene. Exp.: expression; Met: methylation; ND: undetermined; MSI: microsatellite instability; E1: grade 1 endometrioid carcinomas; E2: grade 2 endometrioid carcinomas.

Cases	Type of tumour	*CTNBB1*	*PTEN*	MSI	OTROS FACTORES								
					ARID1A	*PIK3CA*	*KRAS*	Met. *hMLH1*	Met. *hMSH2*	Met. *hMSH6*	Exp. HDAC1	Exp. HDAC2	Exp. HDAC3
4	E1	p.D32N	p.P95T/p.Q111X/p.A126T	ND	---		---	+	+	-	YES	YES	YES
10	E1	p.S37F	p.I253fsX2/p.L320S	ND	---	p.M1043V	---	-	-	-	YES	YES	YES
12	E1	p.T41A	p.I253fsX2/p.G132D	ND	---	p.N1044K	---	+	+	-	No	YES	No
22	E2	p.G34R	p.G132A/p.G165R	ND	p.E2250fsX27	p.H1047R	---	-	-	-	YES	YES	YES
31	E1	p.S37F	p.D300fsX6	ND	p.Q450X	p.H1047R	---	-	-	+	No	No	YES
34	E1	p.G34R	---	ND	---	---	p.G12D	+	+	-	No	No	No
41	E1	p.S33C	---	MSS	p.E1783X	---	---	+	+	+	YES	YES	YES
50	E1	p.S37F	---	MSS	---	p.T1025A	---	-	-	-	No	No	YES
56	E2	p.S45F	p.R130P/p.R130L	ND	---	---	---	-	-	-	YES	YES	YES
68	E1	p.S37F/p.G34R	---	MSI-L	p.T1438fsX6	---	p.G13D	+	-	-	YES	YES	YES
69	E2	p.S37F	---	MSI-L	p.W2091X	---	---	-	-	+	YES	YES	YES

We can say that mutations in the *CTNNB1* gene are mainly accompanied by alterations in the *PTEN*, *PIK3CA*, and *ARID1A* genes. The epigenetic alterations were also frequent but without any correlation with the grade of the tumor. Therefore, mutations in the *CTNNB1* gene would occur in later stages of the tumour development.

The *TP53* gene showed mutations in 11% of the low-grade endometrioid carcinomas analyzed in our work, similarly to that observed in other studies[36]. Four of the six tumours with mutations in the *TP53* gene presented mutations in other genes, mainly in the *PTEN* and *ARID1A* genes (table 40).

Table 40: Genetic and epigenetic alterations presented in those low-grade endometrioid tumours that showed pathogenic mutations in the *TP53* gene. Exp.: expression; Met: methylation; ND: undetermined; MSI: microsatellite instability; E1: grade 1 endometrioid carcinomas; E2: grade 2 endometrioid carcinomas.

Cases	Type of tumour	*TP53*	OTHER FACTORS										
			PTEN	*PPP2R1A*	*ARID1A*	*BRAF*	MSI	Met. *hMLH1*	Met. *hMSH2*	Met. *hMSH6*	Exp. HDAC1	Exp. HDAC2	Exp. HDAC3
26	E2	p.R248Q	p.C136fsX1	---	p.Q1334_R1335dupQ	---	ND	-	-	-	YES	YES	YES
48	E1	p.G266R	p.R335X	p.P179R	p.Q1614fsX20	---	ND	-	+	+	YES	YES	YES
57	E2	p.P151S	---	---	---	---	ND	+	+	-	YES	No	YES
62	E2	p.A223D	---	---	---	---	ND	-	-	-	YES	YES	YES
73	E1	p.R248Q	---	---	---	p.G469A	ND	-	-	+	YES	No	YES
79	E1	p.C135Y	Traslocation?	p.P1175fsX4/p.E1864fsX35	---	---	MSI-H	+	-	-	YES	YES	YES

On the other hand, six low-grade endometrioid carcinomas (10%) did not show any mutations in the analyzed genes. They presented epigenetic alterations but they could have other types of alterations, such as gross arrangements or loss of heterozigosity, or show other pathways or genes altered and recently associated with the development of the endometrioid carcinomas. Is the case of the *PIK3R1* and *PIK3R2* genes, mutated in 20-43% and 5% of the cases, respectively, according to that described in other works[23,260].

Finally, our results show that HDAC2 is the protein with a lack of expression in a higher number of low-grade endometrioid carcinomas (table 25). These results differ from those

observed in other works because, as we have commented above, the HDAC1 protein has been described as the most altered histone deacetylase in low-grade endometrioid carcinomas and therefore, its overexpression, as well as its absence, are indicative of poor prognosis and lower survival of the patients[167,168,244]. Low-grade endometrioid carcinomas are not tumours with a high malignancy and the prognosis of the patients usually is favorable. Therefore, makes sense that the expression of the protein HDAC1 is altered in a low percentage of tumours.

In short, as noted in our work, low-grade endometrioid carcinomas show mutations mainly in the *PTEN*, *ARID1A*, *PIK3CA*, *CTNNB1,* and *KRAS* genes accompanied by MSI, *hMLH1* methylation and loss of HDAC2 protein expression. Moreover, our result suggest that mutations in the *PTEN* and *KRAS* genes, MSI, and methylation of the *hMLH1* gene are early events in the tumour development followed by mutations in the *ARID1A*, *PIK3CA,* and *CTNNB1* genes (figure 29).

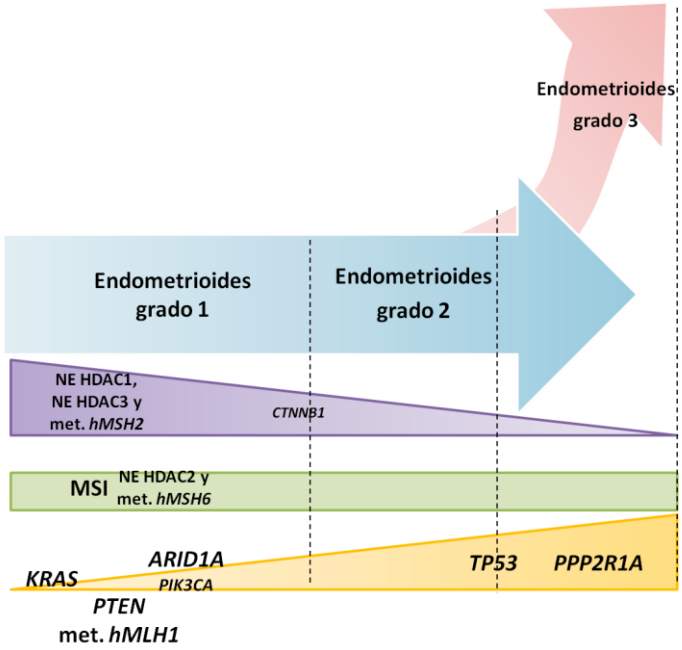


Figure 29: Variation of the genetic and epigenetic alterations analyzed in our work with the increase of the grade of the endometrioid carcinomas. The size of the letter is correlated with the grade of the incidence of the alteration. Met.: methylation; NE: no expression.

4.2 HIGH-GRADE ENDOMETRIOID CARCINOMAS

High-grade endometrioid carcinomas have a clinical behavior similar than non-endometrioid carcinomas[246,247] and can coexist with serous carcinomas. Because of that, many authors have considered that both type of tumours share the same molecular characteristics. However, it has been found that grade 3 endometrioid carcinomas have a more favorable prognosis and molecular characteristics that differ with respect to those of

serous carcinomas. However, the information about the molecular alteration associated with the grade 3 endometrioid carcinomas is limited and it has not been established a molecular profile that characterizes it.

In our work, we analyzed eight grade 3 endometrioid carcinomas (table 41).

Table 41: Genetic and epigenetic alterations presented in the high-grade endometrioid tumours studied in our work. Exp.: expression; Met: methylation; ND: undetermined; MSI: microsatellite instability; E3: grade 3 endometrioid carcinomas;

Cases	Type of tumour	ALTERATIONS SHOWED													
		PTEN	*TP53*	*CDKN2A*	*PPP2R1A*	*ARID1A*	*PIK3CA*	*KRAS*	MSI	Met. *hMLH1*	Met. *hMSH2*	Met. *hMSH6*	Exp. HDAC1	Exp. HDAC2	Exp. HDAC3
9	E3	---	p.R110C	p.A73T	---	---	p.R992X	---	ND	-	-	-	YES	YES	YES
36	E3	p.Y176fsX2	---	---	---	p.S337fsX143	---	---	ND	-	-	-	YES	YES	YES
38	E3	p.P248fsX5	---	---	---	---	---	p.G12V	ND	+	-	-	YES	No	YES
52	E3	p.R130Q p.R142W p.R173Y	p.R175C	---	p.R48Q	p.G154D	p.T1025A p.K1024N	---	MSI-L	+	-	+	YES	No	YES
67	E3	---	---	---	---	p.P146fsX253	---	p.G13D	ND	+	-	-	YES	YES	YES
72	E3	p.R130Q	---	---	---	---	---	---	ND	+	-	-	YES	YES	YES
80	E3	p.G132D p.Q87fsX2	---	---	---	p.N1081fsX11	---	p.G12A	MSI-H	-	-	-	YES	No	YES
82	E3	p.E285X p.T68H	---	---	---	p.P2114fsX20	p.M1043V	p.G12D	ND	+	-	+	YES	YES	YES

In our work, *PTEN* was the gene that was mutated in a larger number of grade 3 endometrioid tumours followed by *ARID1A*, *KRAS* and *PIK3CA* genes (table 46).

The percentage of grade 3 endometrioid carcinomas that showed mutations in the *PTEN* and *PIK3CA* was higher than the low-grade endometrioid carcinomas but without showing significant differences (tables 19 and 31). Some authors have observed, in this kind of tumours, an incidence of mutations in the *PTEN* and *PIK3CA* genes up to 90% and 57% respectively. Our results differ from that described mainly in the *PIK3CA* gene. As we have previously commented, these differences may be due to the low sensitivity of the technique used for the detection of mutations in this gene (CSGE-Heteroduplex), to the largest number of samples analyzed in other works or to the study of exons 1 to 7 of the *PIK3CA* gene where it has been also frequently observed pathogenic mutations[36,57].

We only obtained two blood samples from two patients (cases 52 and 80), which did not allow us to establish a correlation between the MSI and alterations in the MMR. The MMR gene that was methylated in a higher number of grade 3 endometrioid tumours was *hMLH1* as occurred in the low-grade carcinomas (tables 23 and 31). However, we noted again no association between the methylation in the MMR genes and the lack of expression of their proteins.

Our results showed a relation between the *PTEN* gene and the methylation in the MMR genes as we already observed in the low-grade endometrioid carcinomas. 67% of the tumours with the *PTEN* gene mutated showed at least the *hMLH1* gene methylated. Unifying these data with those observed in the low-grade endometrioid carcinomas, we get

that 61% of the endometrioid tumours with mutations in the *PTEN* gene, showed methylation in their MMR genes and, concretely, 55% in the *hMLH1* gene.

KRAS was altered in a high percentage of the grade 3 endometrioid carcinomas (50%), surpassing the rates reported by other authors[36] and the findings in our work about the low-grade endometrioid carcinomas. The different when comparing both groups were significant (tables 19 and 31). This high incidence of mutations of the KRAS gene coincides with the absence of mutations in the *BRAF* and *CTNNB1* genes in this group. This makes sense because, as explained above, according to other authors have reported, mutations in *KRAS*, *BRAF* and *CTNNB1* genes are exclusive[234,254].

On the other hand, mutations in the *TP53* and *PPP2R1A* genes appeared in a percentage higher than in the group of the low-grade carcinomas without showing significant differences (tables 19 and 31) and similar than detected in others works for this subtype[24,36]. Alterations in these genes are typical of non-endometrioid carcinomas and this is one of the main features that differentiate grade 3 endometrioid carcinomas of the low-grade endometrioid carcinomas and invalidate the dualistic theory proposed by Bokhman et *al*.[7].

Finally, in our study HDAC2 was the histone deacetylase with a lack of expression in a largest number of grade 3 endometrioid carcinomas similarly than in the low-grade endometrioid carcinomas.

In short, our results show that grade 3 endometrioid carcinomas present mainly altered the *PTEN*, *ARID1A*, *KRAS*, *PIK3CA*, *TP53,* and *PPP2R1A* genes accompanied by MSI, *hMLH1* methylation and the lack of expression of the HDAC2 protein. Unifying these results with those we obtained in the low-grade endometrioid carcinomas, we observe that when increasing the grade, there is an increase of mutations in those genes and in the methylation of the *hMLH1* gene as well as a decrease of the percentage of tumours with *CTNNB1* altered, hMSH2 methylated and loss of expression of the HDAC1 and HDAC3 proteins. The lack of expression of the HDAC2 protein, the hMSH6 methylation and the MSI do not varies among grades (figure 29).

4.3 SEROUS ENDOMETRIAL CARCINOMAS

Our cohort had eight serous carcinomas of which 50% had pathogenic mutations in the *TP53* gene. These results are consistent with reported in other works in which the incidence of pathogenic mutations in this gene in serous carcinomas is between 50% and 90%[24,99,101,233,261,262] (table 42).

Table 42: Genetic and epigenetic alterations presented in the high-grade endometrioid tumours studied in our work. Exp.: expression; Met: methylation; ND: undetermined; MSI: microsatellite instability; Ser.: serous endometrial carcinoma.

Cases	Type of tumours	*TP53*	OTHER FACTORS								
			PPP2R1A	*CTNNB1*	MSI	Met. *hMLH1*	Met. *hMSH2*	Met. *hMSH6*	Exp. HDAC1	Exp. HDAC2	Exp. HDAC3
19	Ser	---	---	---	ND	-	-	-	No	YES	No
20 (H)	Ser.	p.R273H	---	---	ND	-	-	-	YES	YES	YES
25	Ser.	---	---	---	MSI-H	+	+	+	No	YES	No
29	Ser.	p.G248Q	p.S256T	---	ND	-	-	-	YES	YES	YES
32	Ser.	p.V274A	---	---	ND	+	-	+	YES	YES	YES
49	Ser.	---	p.P179R	p.S37F	MSS	-	-	-	No	No	YES
59	Ser.		p.P179R	---	ND	-	-	+	YES	YES	YES
70	Ser.	p.R175H p.G266R	p.P179R	---	MSI-L	-	-	-	YES	No	YES

Furthermore, note that the *PPP2R1A* gene was mutated in 50% of the serous carcinomas studied, result that also complies as described by other authors in recent studies[51,54,210]. It was common the coexistence with mutations in the *TP53* gene and only one tumour showed alterations in another gene, *CTNNB1.*

In our work, we did not observe any mutation in the *CDH1* and *CDKN2A* genes, commonly associated with the non-endometrioid carcinomas development. Numerous studies have reported a frequent decreased or absence of the E-cadherine protein expression in the serous and clear cell endometrial carcinomas. However, it has been observed that this alteration does not correlate with the mutations rate in the *CDH1* gene. The mechanism that causes it is unclear although it is thought to be due to LOH or gene silencing via promoter methylation[158,159,263]. Similarly, the expression of the p16 protein is frequently altered in serous carcinomas and, especially, in papillary serous type[27,202-206]. Its decrease or loss is associated with more aggressive and poorer prognosis tumours and its origin is mainly attributed to homozygous deletions and promoter hypermethylation of the *CDKN2A* gene.[110,112,114]. For these reasons, it is usual that in our study we have not found mutations in either gene.

Two serous carcinomas in our study (cases 19 and 25) had no mutations in any of the analyzed genes. There is the possibility that other factors present alterations in other genetic factors associated with serous endometrial carcinoma. For example, recent studies carried out by cDNA arrays, have shown that in non-endometrioid carcinomas there is a high overexpression of proteins involved in the regulation of the checkpoints during the mitosis. Is the case of the STK15 protein[264]. Likewise, next generation sequencing has allowed to identify very common mutated genes in endometrial carcinoma such as *CHD4* (17%), *FBXW7* (29%) and *SPOP* (8%). *CHD4* encodes the catalytic subunit of the NuRD complex whie the proteins encoded by *FBXW7* and *SPOP* genes take part of the ubiquitin ligase complex[34].

We only obtained blood samples from three patients (cases 25, 49 and 70) and therefore we did not establish a correlation between MSI and alterations in the MMR mechanism.

However, we did not detect a high frequency of methylation in the MMR genes promoters (table 23) although, unlike endometrioid carcinomas, the MMR gene most methylated in the most of the serous endometrial carcinomas was *hMSH6*.

Finally, our results showed a lack of expression of the at least one of the HDAC proteins in 50% of the serous tumours. None of the cases showed lack of the expression of the three proteins and, unlike we observed in the endometrioid carcinomas, the histone deacetylase that was absent in a larger number of tumours was HDAC1. This fact makes sense if we bear in mind that the serous endometrial carcinomas are more aggressive and poorer prognosis tumours and the lack of expression of the HDAC1 protein has been described as an indicator of poor prognosis and a decreased survival of the patients[167,168,244]

Therefore, serous endometrial carcinomas show a specific molecular profile in which there are frequent mutations in the *TP53* and *PPP2R1A* genes accompanied by epigenetic phenomena such as the *hMSH6* gene methylation and the lack of expression on the HDAC1 protein. It is uncommon the occurrence of mutations in the rest of the genes analyzed in our work and it would be appropriate to include the study of new molecular factors as well as the pursuit of other types of alterations (amplifications or LOH) for a complete study of the tumour pathogenesis. Moreover, to increase the sample size and obtain a greater number of peripheral blood samples would allow us to corroborate the epigenetic findings observed in our study.

4.4 CLEAR CELL ENDOMETRIAL CARCINOMAS

Up to now, clear cell endometrial carcinomas had been included in the same category than the serous endometrial carcinomas because both of them are high-grade carcinomas with an aggressive behavior. However, it has been observed that they are no comparable each other due to they have very different clinical, immunohistochemical and molecular characteristics[265].

In our work, we studied a clear cell endometrial carcinoma (case 30) (table 43).

Table 43: Genetic and epigenetic alterations presented in the clear cell endometrial carcinoma studied in our work. Exp.: expression; Met: methylation; ND: undetermined; MSI: microsatellite instability; CC: clear cell endometrial carcinoma.

Case	Type of tumour	ALTERATIONS SHOWED								
		ARID1A	*hMLH1*	MSI	Met. *hMLH1*	Met. *hMSH2*	Met. *hMSH6*	Exp. HDAC1	Exp. HDAC2	Exp. HDAC3
30	CC	p.G276fsX86	IVS3+5G>A	ND	-	-	-	YES	No	No

As we did not have the blood sample of the patient, we could not check if it was an endometrial carcinoma associated to Lynch syndrome.

Our results are not comparable but are in concordance with what has been described about 35% of the clear cell endometrial carcinomas have mutations in the genes implicated in the chromatin remodeling and in the ubiquitin ligase complex[34]. The protein BAF250a is encoded by the *ARID1A* gene and takes part of the SWI/SNF complex that is implicated in the ATP-dependent chromatin remodeling. In our case, it showed a frameshift mutation previously described by other authors[176]. Other series have observed a loss of the BAF250a expression in 26% of the clear cell endometrial carcinomas, rate that exceeds that of the serous (18%)[227]. However, mutations detected in the *ARID1A* gene in both subtypes are very rare. Moreover, it has been described other genes associated with the clear cell endometrial carcinoma that are also implicated in the serous carcinomas: *FBXW7,* mutated in 13% of the cases; *SPOP*, in 9%; and *CHD4,* in 4%[34].

In the clear cell endometrial carcinomas it has been also described mutations in *TP53,* (9%)[266] and loss of the E-cadherine protein expression as a result of a LOH in the *CDH1* gene[263,267]. However, the presence of a mutation in the *hMLH1* gene results unusual given that in the sporadic endometrial carcinoma is not very common the appearance of mutation in the MMR genes[138].

Respecting the loss of the HDAC2 and HDAC3 proteins expression there is nothing described about it. It has only observed that the overexpression of the three histone deacetylases is more frequent in serous and clear cell endometrial carcinomas than in endometrioid carcinomas[168].

4.5 MIXED ENDOMETRIAL CARCINOMAS

Mixed endometrial carcinomas have a very heterogeneous profile and because of that, their molecular characteristics are very mixed depending on their composition.

In our work, we analyzed two mixed endometrial carcinomas. In the tumour 23, the 50% was a grade 2 endometrioid component and the other 50%, clear cell and serous carcinoma component. A mix of a grade 3 endometrioid carcinoma and one serous carcinoma formed the tumour 66.

The characteristics that they showed were similar than the endometrioid carcinomas ones (table 44).

Table 44: Genetic and epigenetic alterations presented in the mixed endometrial carcinomas studied in our work. Exp.: expression; Met: methylation; ND: undetermined; MSI: microsatellite instability.

Cases	Type of tumour	ALTERATIONS SHOWED									
		PTEN	*ARID1A*	*KRAS*	MSI	Met. *hMLH1*	Met. *hMSH2*	Met. *hMSH6*	Exp. HDAC1	Exp. HDAC2	Exp. HDAC3
23	Mixed	p.R130G	p.E1779fsX3 p.P1326fsX11	p.G13D	MSI-H	-	-	-	YES	YES	YES
66	Mixed	---	p.N106fsX5	---	ND	-	-	-	YES	YES	YES

The tumour 23 showed a molecular profile identical to described in the low-grade endometrioid analyzed in our work in which there is a coexistence of mutations in the *PTEN*, *ARID1A,* and *KRAS* genes accompanied by MSI. This result suggests that in this case the endometrioid component disguises the non-endometrioid component though the mutations in the *KRAS* and *ARID1A* genes could be provided, but with a lower probability, by the serous component.

In the tumour 66, we only observed a mutation in the *ARID1A* gene. This fact results unusual because it is a tumour with high-grade components, which usually show a high number of mutations.

In both tumours, it would be convenient to increase the study and analyze new genes such those already mentioned associated to the development of the endometrioid and serous carcinomas.

In short, mixed carcinomas are high-grade tumours with a bad prognosis and molecular characteristics typical of their components. To characterize them, it would be convenient to make first the anatomical-pathological study and, once described their composition, to analyze those molecular factors associated to the development of the carcinomas that integrates them.

4.6 CARCINOSARCOMAS

Carcinosarcomas are very similar to the grade 3 endometrioid carcinomas but it has been observed differences in its clinical behavior. Patients with carcinosarcomas show a worse prognosis with a low survival rate. These variations can be accompanied by molecular differences.

Our cohort had 12 carcinosarcomas (14% of the total endometrial carcinomas studied) with a heterogeneous composition and in several cases we could not obtain enough information about the characteristics of their components (table 45).

Table 45: Genetic and epigenetic alterations presented in the carcinosarcomas studied in our work and their epithelial component. Exp.: expression; Met: methylation; ND: undetermined; MSI: microsatellite instability; E3: grade 3 endometrioid carcinomas;

Cases	Epithelial component	ALTERATIONS SHOWED											
		PTEN	*TP53*	*ARID1A*	*PIK3CA*	*CTNNB1*	MSI	Met. *hMLH1*	Met. *hMSH2*	Met. *hMSH6*	Exp. HDAC1	Exp. HDAC2	Exp. HDAC3
5	Endometrioid	p.R172fsX5	---	p.G889fsX2 p.D1850fsX32	---	---	ND	+	+	+	Yes	Yes	Yes
11	?	---	p.S241F(LOH)	---	---	---	ND	-	-	-	No	Yes	No
16	Serous	---	p.C176fsX180	---	---	---	MSI-H	-	-	-	Yes	Yes	Yes
24	Endometrioid	---	---	---	---	p.T41A	ND	-	-	+	Yes	Yes	Yes
27	Serous	p.Q171H (H)	p.P153fsX179 p.P278S	---	---	---	ND	-	-	-	Yes	Yes	Yes
39	?	p.E256X	---	p.I1173fsX6 p.Q2115X	---	---	ND	-	-	-	Yes	Yes	Yes
46	Grade 1 endometrioid	---	---	---	p.M1043V	---	ND	-	-	+	Yes	Yes	Yes
53	Serous	---	p.N239D	---	---	---	MSS	+	+	+	Yes	Yes	Yes
58	Epidermoid	---	p.R213X	p.S1000Y	p.M1043I	---	ND	-	-	-	Yes	No	Yes
63	Grade 3 endometrioid	p.344delK	---	---	---	---	ND	-	-	+	No	No	Yes
81	Serous	---	---	---	---	---	ND	-	-	-	No	No	Yes
84	Grade 3 endometrioid	p.W274X	p.R249S	p.D1850fsX32	---	---	ND	+	-	+	Yes	No	Yes

We observed that every tumour with endometrioid component (cases 5, 24, 46, 63, and 84) showed mutations in those genes associated with endometrioid carcinomas: *PTEN*, *PIK3CA*, *ARID1A,* and *CTNNB1*. Similarly, all the Carcinosarcomas with serous component excepting one (cases 16, 27, and 53 but not 81), presented mutations in the *TP53* gene, associated to serous endometrial carcinomas. This correlation has already described y other authors who have proposed the existence of two types of carcinosarcomas: carcinosarcomas with a mutation pattern similar than the endometrioids carcinomas profile, with mutations in the *ARID1A*, *PTEN, PIK3CA,* and *KRAS* genes; and the carcinosarcomas with a mutation pattern similar than serous carcinomas profile, with mutations in the *TP53* and *PPP2R1A* genes[36].

The tumour 81 was a exception because despites its serous epithelial component, it did not show any mutation in the analyzed genes. It would be convenient to study other genes implicated in the development of the seros carcinomas such as *STK15, CHD4, FBXW7,* and *SPOP*[34].

The sample 58 was a carcinosarcoma with epidermoid component, instead an adenocarcinoma, and mutations in the *TP53, ARID1A,* and *PIK3CA* genes. This kind of tumours is very infrequent maybe due to its keratinocytic origin, can have a different molecular profile. It has been descried that the *PIK3CA* gene is mutated in a 9% of the epidermoids head and neck tumours[268].

Similarly than observed in the serous carcinomas, *hMSH6* was the MMR gene most methylated in the carcinosarcomas, even in those with endometrioid epithelial component.

On the other hand, the histone deacetylase that showed a loss of expression in a highest number of the studied carcinosarcomas was HDAC2 followed by the HDAC1 and HDAC3, similarly than endometrioid carcinomas.

Therefore, the results observed in our work show that carcinosarcomas have a genetic profile that depends on their epithelial component whereas, the epigenetic alterations do not adjust to any already described histological pattern. Being tumours that are in a constant transformation process, they could be subjected to permanent changes in their epigenetic mechanism because the evolution of their cells from epithelial cells to mesenquimal cells requires an permanent activation and silencing of the gene transcription (figure 30).

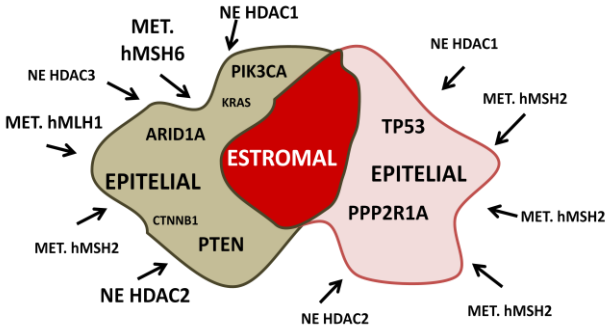


Figure 30: Molecular alterations associated with endometrial carcinosarcomas depending on the histological characteristics of their components: serous on the right, endometrioid on the left. The size of the letters is correlated with the incidence grade of the alteration. MET.: methylation; NE: no expression.

In view of our results, the most appropriate classification for the determination of the molecular profiles in sporadic endometrial carcinoma is one that takes into account the molecular characteristics specific of each histological subtype. The classifications according to the clinical-pathological criteria and histological grades are not specific and incorrect because they group types of tumours with different genetic and epigenetic profiles. Classification by histological subtypes results more accurate and allows to obtain more detailed information about the molecular profile, facilitating in this way the search of more adequate treatments.

The new molecular techniques allow the study of a large number of factors implicated in the tumorigénesis. In order to find more effective therapies to combat tumour development, it is essential to know those processes that are altered; recent studies, carried out by genomic and proteomic analysis, have proponsed a different classification different than those discussed in our work. It divides endometrial carcinomas en four groups: *POLE* ultramutated, microsatellite instability hypermutated, copy-number low, and copy-number high[269].

Future studies will focuse on the optimization of this classification checking its uselfullness when looking for specific treatments for the different types of sporadic endometrial carcinomas.

5. THE TELOMERES ANALYSIS IN SPORADIC ENDOMETRIAL CARCINOMAS

In our work, we analyzed the telomere length in the different types of tumours, grades and histological subtypes. The group of endometrial tumours of clear cells was removed from the study because it had just one sample.

When comparing the three tumour grades we observed that their telomere lengths did not significantly differ. However, when comparing by histological subtypes, grade 3 endometrioid carcinomas showed a telomere length strongly lower, but not significantly different, than the grade 1 and 2 endometrioid carcinomas groups. This fact could suggest that telomerase complex could be silenced, instead overexpressed, when the grade of the tumours increased causing higher chromosome instability that would be accord with a higher aggressiveness and worse prognosis of the grade 3 endometrioid tumours. Grade 3 endometrioid tumours have molecular characteristics halfway between low-grade endometrioid carcinomas and serous endometrial carcinomas. However, in both cases their telomere length was lower although the serous endometrial carcinomas are also high-grade tumours.

Several authors have elaborate the theory of the telomerase overexpression associated with longer telomeres and larger cell survival[195]. According to this, we could think that non-endometrioid carcinomas should show longer telomeres; however, it has been described a telomere shortening in the non-endometrioid carcinomas development when it exists the inactivation of at least one of the alleles of the *TP53* gene. Experiments carried out in mice have showed that when both alleles of the PTEN gene are inactivated, there is a development of endometrioid carcinomas[270]. However, the inactivation of one of the alleles of the *TP53* gene does not cause the development of non-endometrioid tumours[271] so, therefore, it is necessary the participation of more factors that give rise to that development. As the main function of the telomeres is the maintenance of the chromosome stability that is altered in the tumour development[272], it has been proposed the telomere shortening, with its consequence telomere vulnerability, as another of the triggering factors of the tumorigénesis in the non-endometrioid carcinomas[273]. In our study, we have observed similar telomere length in the endometrioid and non-endometrioid carcinomas. Therefore, our results do not support the aforementioned theory.

Mixed carcinomas are tumours consisted of endometrioid and non-endometrioid components. In our work, they showed telomeres shorter that the rest of the groups excepting the grade 3 endometrioid carcinomas.

On the other hand, the carcinosarcomas, which are the most aggressive and with a worst prognosis endometrial carcinomas, did not show significant differences in their telomere length when comparing them with the rest of the groups.

The tDNA forms two different strands. The 5´-3´ strand is denominated G-strand because there is a high percentage of Guanines while the 3´-5´ strand in enriched in Citosines. The G rich strand is not matched with the other strand forming a unpaired segment that constitutes the 5´extreme of the chromosome. It adopts a buckle secondary structure stabilized by many proteins such as POT1, TRF1, TRF2, TIN2, RAP1, TNKS2, or TPP1[182,183,274,275,276,277].

Several studies have analyzed the possible implication of POT1, TNKS2, TERF1, TERF2, and TERT proteins polymorphisms in a lower protection of the telomere extremes and therefore, in their high degradation. It has been only found a correlation with the rs2736122 (*TERT*) and rs12412538 (*TNKS2*) polymorphisms[278]. However, every study confines to analyze the risk of developing endometrial carcinoma without bearing in mind the subtype or malignance grade. In our work, we have analyzed two polymorphisms associated with a telomere shortening and the consequent loss of the chromosome stability: *TERT-1327C>T* and *TERC-63G>A*. Concretely, the alleles C and G, respectively, have been related to a lower activity of the telomerase enzyme due to a decrease of the promoter activity when they are present[220,221].

In our study we analyzed the differences in the genotypes distribution depending of the types of tumours established by the clinical-pathological classification and the histological grades and subtypes. We only observed significant differences in the genotype distribution in the *TERT-1327T>C* polymorphism when we compared all the subgroups contained in both clinical-pathological and histological classifications. The C/C genotype appearance frequency was higher in the non-endometrioid carcinomas and increased with the grade finding high levels in the highest-grade histological subtypes. On the other hand, this increase was associated with a decrease of the C/T genotype appearance frequency. Given that the allele C has been related to a lower activity of the telomerase, we grouped tumours by alleles. When we grouped those genotypes that contained the allele C, we did not observe significant differences when comparing neither endometrioid with non-endometrioid carcinomas nor among the different grades. However, when grouping the genotypes with the allele T, we found significant differences between the endometrioid and non-endometrioid carcinomas groups. In the non-endometrioid group, the percentage of tumours that presented the genotype C/C was higher than the percentage of tumours that contained the allele T (T/T+C/T). This fact did not occur in the endometrioid carcinomas and because of that, there was significance when we compared

both groups. Therefore, although the allele C has been associated with a lower activity of the telomerase[220], our results showed that is the genotype C/C which is associated with non-endometrioid carcinomas, more aggressive tumours and with a worse prognosis, but with a lower telomere length.

When we grouped the genotypes containing the allele C (C/C+C/T), we only found significant differences when we compared the mixed carcinomas with the grade 2 and with the serous carcinomas. This result adjusts to the lower telomere length detected in the mixed carcinomas group although it has only two cases, it would be convenient to increase the sample size in order to corroborate it.

The genotype distribution of the *TERC-63G>A* polymorphism did not show significant differences. We only observed a significant p-value when we grouped the allele G (G/G+G/A vs A/A) and we compared the carcinosarcomas with grade 3 endometrioid carcinomas. In a 75% of the grade 3 endometrioid carcinomas, the allele G was presented while in the rest of the groups, the allele A was the most frequent. Given that allele G has been associated with a lower activity of the telomerase, these results could be related to the shorter telomeres showed by the grade 3 endometrioid carcinomas.

In conclusion, in our work we have not observed a clear correlation between the telomere average length and the different types of tumours, observing that only the grade 3 endometrioid and the mixed carcinomas showed shorter telomeres than the rest of the groups. The analysis of the *TERT-1327C>T* and *TERC-63G>A* polymorphisms showed a slight association between the allele C and the mixed carcinomas as well as between the allele G and the grade 3 endometrioid carcinomas.

Finally, it would be convenient the study of certain epigenetic aspects in the telomeric and subtelomeric regions. Recently, it has been showed that the methylation and deacetylation of the histones located in these regions constitute an important repressor of the tDNA recombination[279,280]. When there is a low methylation and/or a high acetylation in the subtelomeric and telomeric regions, the telomeric chromatin is “open” and allows that the telomerase and the proteins access easier to the structure to elongate the telomeres[280]. Bearing in mind that the tumours are subjected to frequent epigenetic changes, this hypothesis results feasible and these events could play an important role in the tumour development.

CONCLUSIONS

1. The results of our study confirm the high incidence of the pathogenic mutations in the *PPP2R1A* gene in serous sporadic endometrial carcinoma, though, as it appears mutated in other histological groups, it is suggested the necessity of studying this gene in other tumour subtypes

2. The demonstration of mutations in different exons of the *PPP2R1A* gene justifies its full study, instead the analysis of only some selected exons, to perform a correct molecular diagnosis. On the other hand, our work shows for the first time that in some endometrial tumours it is detected larger PPP2R1A protein variants, probably because of a posttranscriptional processing, and therefore, it would be convenient further analysis of these variants to determine their possible role in tumour development.

3. The *ARID1A* gene shows a high rate of pathogenic mutations in sporadic endometrial carcinoma without observing hotspots, being necessary its study in routine genetic studies of these tumours.

4. The telomere length of the sporadic endometrial carcinomas is not associated with the different histological grades and subtypes.

5. In our study, the microsatellite instability and the lack of the hMLH1, hMSH2, and hMSH6 proteins expression, detected by immunohistochemistry, do not correspond to the presence of mutations, gross rearrangements and methylation of these MMR genes. Therefore, it seems to be necessary the presence of other genetic or epigenetic factors that can be implicated in the appearance of these alterations in sporadic endometrial carcinoma.

6. In our work, we have observed that the mutations in the *PTEN* gene correlate with the methylation of the MMR genes and mainly with the *hMLH1* gene in the endometrioid carcinomas, suggesting that alterations in the DNA repair systems favors mutations in the PTEN gene in this type of tumours.

7. Finally, our analysis of the molecular alterations in sporadic endometrial carcinoma shows that of the different classifications purposed so far, the most appropriate, accurate and specific is one that takes into account the histological subtype of the tumour and its molecular alterations. This confirms that histological and molecular analysis of the tumour provides information about its progression and they will allow designing specific and targeted treatments.

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