Microsatellite Instability (MSI) as Genomic Marker in Endometrial Cancer: Toward Scientific Evidences

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Abstract: Endometrial Cancer is the most frequent tumor in western world nations, with 142,000 new cases each year and 42,000 casualties. This form of cancer typically affects women between 55 and 65 years of age, and ranks fourth among female tumors.

Endogenous predisposing conditions to endometrial cancer development are: late menopause, early menarche and hyperestrogenism, while hormone replacement therapy, obesity, alcohol, diabetes, and a diet rich in animal fats as well as chronic liver disease, are the exogenous factors.

This tumor may also have an hereditary predisposition, as in the Lynch Syndrome or in HNPCC (Hereditary NonPolyposis Colorectal Cancer), since genetic modifications induced by the "MisMatch Repair" genes lead to a tumoral development susceptibility, not only in the colon.

The phenotypical consequences of these genetic modifications may be found in the microsatellite instability (MSI) and in the loss of heterozygosity (LOH), which generate the replication errors in positive phenotypes repeats. These express the incapability to repair short nucleotide insertions or deletions, generated by a wrong DNA replication. Due to such genetic modifications, new allelic variants arise in the endometrial tissue, confirming the high degree of this genetic disorder.

Recent studies showed that the MSI and LOH in endometrial cells may be associated with the possible loss in the expression of cellular control and with the possible degeneration of the cell growth phenomenon.

There is also a possibility of utilizing these new genetic markers in the endometrial mucosa to study these tissues and to detect any possible neoplastic transformations, thanks to Genomics.

Keywords: Endometrial cancer, microsatellite instability, MSI, HNPCC, endometrial hyperplasia, Lynch sindrome, genomics, proteomics, laparoscopy, endoscopy, gynecological cancers, malignant uterine cancers, uterine tumors.

INTRODUCTION

The endometrial carcinoma is the most frequent gynecological tumor in developed countries. It ranks fourth among female malignant tumors, affecting the glandular endometrial epithelium, causing a fast increase in cellularity. Extrauterine endometrial cancer usually spreads *via* a lymphatic approach, albeit often locally to the vagina, tube and ovaries [1]. The endometrial carcinoma may have an onset either due to hyperestrogenism or sporadically, yet it will often have an hereditary predisposition, like in the Lynch syndrome or in the Hereditary NonPolyposis Colorectal Cancer (HNPCC) [2].

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Genetic alterations in some genes, called “MisMatch Repair genes” (MMR), are, in fact, the main cause of the HNPCC. This disorder affects the colon as well as the other organs such as the which endometrium, the stomach, the ovaries, the brain, the hepatobiliary epithelium and the uro-epithelial epithelium. In recent years, the genes responsible for the susceptibility to HNPCC have been identified [3]. The MMR genes are: hMSH2, hMLH1, hPMS1, hPMS2 and hMSH6, as reported on Table **1** [4-12].

Once genetically modified, each of these generates a phenotype that predisposes the individual to cancer [13].

The phenotypic consequence of the aforesaid mutations in MMR genes is represented by microsatellite instability (MSI) and by the loss of heterozygosity (LOH), both generating a so-called phenotype with “replication errors in repeats” (RER) or RER positive [13].

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Table 1. MMR Genes Associated to Germline Mutations with Endometrial Cancers and Other Extra-Colic Cancers

Gene	Phenotypic Features Associated to Germline Mutations	References
MLH1	Typical of HNPCC. Different phenotypic manifestations.	[4-6]
MSH2	Typical of HNPCC. Involved mainly in Muir-Torre syndrome and in extra-colic tumors more than MLH1 gene.	[5,7]
MSH6	Frequently mutated in endometrial cancers and in colorectal cancers, more distantly.	[8,9]
PMS2	Involved mainly in Turcot syndrome, and having different genetic relevance	[10,11]
MLH3	Generally not associated to HNPCC. Sometimes involved in tumors in colorectal cancers, more distantly.	[12]

Abbreviations: *HNPCC, Hereditary NonPolyposis Colorectal Cancer.*

Such phenotype expresses, therefore, an inability to repair small insertions or deletions of nucleotides, caused by not corrected DNA replication. Such genetic alterations, thus, generate, in turn, a new variety of alleles in somatic tissues, confirming the elevated degree of the genetic disorder [14, 15].

The MSI shows, moreover, a significant association with myometrial invasion and an advanced stage disease, although the impact of MSI on endometrial carcinoma survival is still causing lively debates [14].

Microsatellite: General Features

As underlined above, the cause of the carcinogenesis is to be found in the ongoing accumulation of genetic mutations, due to lack of control during cell replication. Such mutations involve, either an altered enzyme activity codified by repair genes, or they seem to be responsible for the predisposition to such hereditary neoplasms.

One of the consequences of the above mentioned DNA mutations, easily detectable in the lab, is the appearance of MSI. Tumors and adenomas developed within the HNPCC, show MSI features [16].

In the human genome there are approximately 500.000 microsatellites loci, tandem-repeated, with a variable number of nucleotides (minimal base). Thus, the variations in the length of microsatellites, due to insertions or deletions of single units, may be defined as MSI [17].

In the description of a microsatellite, important sequences appear also "flanked": these are only sequences placed side by side, thus enabling the design of primers that promote the amplification of microsatellite [17].

MSI is the consequence of defects in the "*system of repair of the wrong pairing*", i.e. the mismatch repair. In the human genome, the systems of repair of the wrong pairing are represented by genes codifying for six proteins: hMLH1, hMLH3, hMSH2, hMSH3, hMSH6, hPMS1 and hPMS2. Such proteins identify and eliminate anomalous DNA sequences formed during replication processes, due to the effect of chemical or physical agents.

Microsatellites are genetic regions with a high mutation risk. The genes most prone to mutations in microsatellites are: TGFβRII, E2F-4, BAX and IGFIIR [18].

While defects in MLH1 and MSH2 generate mainly an instability in the di-nucleotide repetitions, defects in MSH6 usually show alterations in mononucleotide repetitions. Such findings find a confirmation in the biological functions of such genes. In fact, while MLH1 and MSH2 repair also errors of insertions or of deletions of 2-4 bases, MSH6 is involved in the repair of single substitutions and the repair of insertions or deletions of single bases [19].

Use of MSI in the Identification of Tumors

MSI analysis can concur to the identification of subjects and families that are likely to be carriers of germline mutations in MSH2 or MLH1. The research in MSI must also focus on the need to distinguish a Familiar Adenomatous Polyposis (FAP), attenuated by the HNPCC.

The FAP is an hereditary form of colorectal cancer that, above all, turns out to be the most common cause of death after the lung cancer and breast cancer in women, and after lung and prostate cancer in man, in the populations of developed countries [1-3].

Some researchers clarified the genetic bases of hereditary forms of FAP and of HNPCC, in which alterations of a single gene, transmitted through the germline cells, cause a marked familiar predisposition to the development of the colorectal tumor.

Either in FAP or in HNPCC, the tumor appears in individuals with a mean age of 42, approximately 25 years before the average age of onset of the sporadic colorectal tumor. Nevertheless, while in the FAP the molecular defects generate the tumor, in HNPCC the molecular defects trigger the progression of the tumor [20].

The National Cancer Institute (NCI) has recently drafted guidelines for the identification of MSI in the colorectal carcinoma, involving the study of two genetic loci comprising two mononucleotide repetitions (BAT25 and BAT26) and three loci of di-nucleotide repetitions (D2S123, D5S346 and D17S250).

Yet, instability is defined as a condition in which, in a cell population, there are cells that show, in their DNA, a variable number of genetic repetitions.

Cancers can be thus classified as high-degree MSIs or MSI-H (instability shown in more than 30-40% of the loci studied), low-degree MSIs or MSI-L (instability shown in less than 30-40% of the loci studied), in cancers with MSS (stability of microsatellites) [1-3].

Tissue analysis MSI on neoplasms and/or on polyps is considered a first-level genetic analysis, in order to characterize subjects potentially prone to HNPCC. They will subsequently be screened for MSH2 or MLH1, as shown on Table **2** [21-25].

Analysis of MSI in Tissues

The human genome is subdivided in:

- Not-interspersed DNA
- interspersed repetitive DNA
- Tandem-repetitive DNA (or satellite DNA)

Not-interspersed DNA accounts for approximately 46% of the entire genome; it is made up of the codifying regions, which represent only a small portion of the genome, as well as of the non-expressed regions, involved in the regulation of the transcription, in the maintenance of chromosomic structural integrity [26].

The interspersed repetitive DNA, instead, represents approximately 45% of the nuclear genome and comprises the LINEs, SINEs elements, the retro-elements LTR and the DNA transposons.

The Tandem-repetitive DNA, or satellite DNA, includes the α and β satellite DNA, the minisatellites and the microsatellites. This distinction is linked to the size of each single unit of repetition and to the total length of the sequence (although they exist, considering the discordances among all the authors on the size of every subgroup) [27].

The satellite DNA or TRS (*tandemly repetitive sequence*) is made up by nucleotide sequences, that are repeated exactly in tandem.

The term satelliteDNA derives from the fact that, based on its various density, it can be separated from the remaining DNA by means of technical details of centrifugalization [27]. TRSs are widely interspersed along all the genome and are polymorphic, i.e. in same locus a number of repetitions of the sequence will vary from an individual to the other. Due to these features, TRSs are used like markers for the construction of genomic maps or linkage maps [26].

Satellites are the first TRS discovered; the single unit of repetition can expand to numerous hundreds of bases, while the total size is comprised between 100 kb and several Mb [26].

Satellite DNA, most often found in the heterochromatinic centromeric areas, is not transcribed nor does it have any biological function. Satellites cannot be employed like markers in "*DNA profiling*" because they are far too large in size, which is a constraint limiting the possibility of amplification by means of the *Polymerase Chain Reaction* (PCR) technique. Moreover, their almost exclusive localization in some areas of the genome is a non-eligibility factor in genetic linkage studies [27].

Minisatellites, General Features

Minisatellites are nucleotide sequences smaller than the satellites, with a size between 100bp and 20kb. In minisatellites, the size of the repetition unit ranges between 6 and 50bp.

Moreover, minisatellites differ from satellites as they have major biological functions, such as the corrected replication of telomeres, as shown by the group of the so-called the telomeric minisatellites. These protect chromosome endings from degradation processes. In more general terms, the minisatellites are characterized by a high degree of polymorphism and therefore have all the ideal characteristics to be used as genetic markers in "DNA profiling", and, consequently, in the paternity exclusion test [26, 27].

Table 2. Investigation of MMR Genes Responsible for MSI in Patients with Colorectal Cancer

MMR Gene Studied	No. Patients	Proportion of Colorectal Cancers with Alterations in these Genes	Genetic Approach	References
MLH1	46	36/46	–Immunohistochemical analysis –Methylation analysis	[21]
MSH2		7/46		
MLH1	51	48/51	–Sequencing (MLH1, MSH2, MSH6) –Methylation Analysis (hMLH1 and hMSH2 promoter)	[22]
MSH2		3/51		
MSH6		4/51		
MLH1	57	34/57	–Sequencing (MLH1, MSH2, MSH6) –Immunohistochemical analysis	[23]
MSH2		22/57		
MSH6		1/57		
MLH1, MSH2	58	3/58	Sequencing (MLH1, MSH2)	[24]
MLH1, MSH2, MSH6, PMS2	185	13/185	–Sequencing (MLH1, MSH2, MSH6, PMS2) –MLPA (MLH1, MSH2) –Methylation studies (MLH1 promoter)	[25]

Abbreviations: *MLPA: Multiplex Ligation-dependent Probe Assay.*

From a historical point of view, minisatellites, discovered in 1985 at the University of Leicester [28], have been employed in DNA finger-printing before the discovery of the even smaller microsatellites. They have replaced, in fact, in most DNA "profiling" analyses. Moreover, although their high level of polymorphism, minisatellites are not eligible in genetic linkage studies as they stretch out and are mostly located at chromosome endings [29].

Microsatellites in Tumoral Studies

Conversely, in order to detect early-stage neoplasias, markers should be homogenously distributed in the entire genome.

Polymorphism of satellites and of minisatellites seems to be ascribed either to irregular crossing phenomena between chromosomes, or to irregular crossing phenomena between chromatidia siblings, which occur every hundreds or thousands of years [16].

Microsatellites or short tandem repeats (STRs), represent the most useful genetic marker.

STRs are short DNA sequences composed of mono, bi, tri, tetra or pentanucleotide units repeated in tandem, with a total length of the entire sequence ranging from tens to hundreds of bases [30].

Microsatellites have a much lower allelic mutation rate during meiosis, thus being sufficiently stable to be employed like genetic markers for Mendelian inheritance [29].

STRs are dispersed in the genome with a frequency inversely proportional to the length of their repetition unit. Mononucleotide repetitions will, therefore, appear in the genome several million times, whereas pentanucleotides only some thousands of times. Larger nucleotide sequences might appear less frequently taking account of the way microsatellites are formed --a process which numerous authors ascribe to the phenomenon of "*polymerase slippage*" or "*slipped strand mispairing*" [30].

The slippage is an error performed by the DNA Polimerase because of an imprecise pairing, generally of a repetition unit, between the filament stamp and the newly-synthesized filament. Consequently, the region not paired is forced to form a fork, a "loop out", which will be excluded from the replication process.

If there is a "*loop*" on the filament stamp, there will be a "*slippage forward*" on the newly-synthesized filament that is being expanded, with a clear deletion of a repetitive unit [29].

Conversely, if a fork is formed on the expanding filament, the end result will be a "*slippage backward*" with the consequent insertion of a repetitive unit on the newly-synthesized filament.

In more general terms, observations have shown that the frequency with which the tetra- and pentanucleotide microsatellites appear along the genome, drops probably because the formation of loops having a 4 or 5- nucleotide size is more unstable and, therefore, less eligible as far as forks are concerned than the single two nucleotides. The theory of the formation of STRs by means of "*polymerase slippage*" would imply a limitless increase of the very microsatellites in time, but this process physiologically turns out to be limited, as it occurs at the same time a tendency to error repair during replication [16].

Indeed, many forks that have randomly been formed, are subsequently eliminated by cellular repair systems; hence, the coexistence of two phenomena due to "*slippage*" processes, stretching out to modify the sequence, as well as to other reparative phenomena.

It is fundamental to specify that, when "*slippage*"occurs, a mutational event takes place. Actually, the frequency of the "*slippage*" phenomenon is much greater than the STRs' mutation rate and this is due to the reparative processes that correct the error caused by "*slippage*" [26, 27].

The mutation rate is therefore determined by the final outcome of a mutual game between errors of replication and successive corrections. This balance between opposite phenomena appears fortunate to the eyes of geneticists. Indeed, if the mutational phenomena were to take place rarely, this would involve the presence of a low number of microsatellites associated to a low degree of polymorphism. On the other hand, if the formation of forks, and the consequent slippage were not repaired, a variability of the microsatellites also from a parental generation to the progeny would take place.

If, therefore, by means of a parental analysis with microsatellites, a child had a pattern of incompatible heredity with that of his parents, he might most probably be an adopted child instead of being generated by a mutational event [16].

Microsatellites in the Prokaryote: Functional Meaning

Before considering how microsatellites are currently used in scientific studies on the eukaryotic genome, it is worth underlining fact that STRs are present also in inferior organisms which, among other things, have proved to have interesting biological functions. By means of example, in any bacterial population there are genes, called contingency genes, characterized by the fact they are expressed intermittently and by the fact that they contain microsatellites, within their sequence, [29, 30].

Such genes guarantee the survival of the bacterial population, acclimatization to factors such as in the occurrence of Neisseria gonorrhoeae, whose contingency genes help the bacterium elude the immune system.

The Neisseria has at least a dozen of genes codifying for external membrane proteins, genes characterized by repetitions of unstable pentanucleotide units. Should "*slippage*" occur in a number of nucleotides greater than 3 -the number of nucleotides that identifies a "codone"- this would cause a "*frameshift*", i.e. a variation of the nucleotide reading frame (ORF, open reading frame) formed by all triplets codifying each for an aminoacid. The sliding of the reading frame of a number of nucleotides other than 3, usually involves the complete loss of the DNA codifying ability, thus determining a repression of the expression of that protein [29, 30].

Analogously, another slippage restoring the ORF will determine the recovery of the expression of that protein. The

result of this dynamic process involving, simultaneously, the expression of some surface genes and the repression of others, ensures the introduction of the bacterium into the host's immune system, with an antigenic constellation of various surface genes from one generation to the other. Such phenomenon is known as phase variation [16].

Functional Meaning of Microsatellites in the Eukaryotes

Although the contingency genes seem to be limited to the prokaryotic genome, it would be surprising to find that microsatellites do not have a biological role also in advanced organisms, due to the high number and common presence inside the same codifying sequences.

There are pole triplets, in fact, that are equal between them, found in genes of eukaryotic organisms, codifying for pole-glutamic sequences, which seem to be accountable for the role of the transcription regulation [26, 27].

Consequently, moving up along the biological scale, the role of repeated sequences has taken on an even more sophisticated meaning than that of contingency genes. In prokaryotes, in fact, STRs act as on-off signals because they allow or prevent the expression of the genes in which they are located, while in eukaryotes these sequences act like "switches", or gene expression regulators [29].

Moreover, microsatellites may also have negative biological roles and seem to be responsible for some genetic diseases, such as Huntington's disease, and the X Fragile Syndrome in men.

The pathological process is linked to the expansion of trinucleotide repetitions, which are responsible for the name of "*tri-nucleotide repeats associated diseases*". Such diseases show a direct relationship between the pathological severity and the anomalous extension of the triplets, which, moreover, is enhanced from one generation to another, giving rise to an increasingly earlier onset and symptom severity [30].

The absence of diseases associated with trinucleotide sequences in other animals, including the ones closer to man in the evolution scale, have lead to important considerations. The triplets repeated in the genes of neurons may have led to an evolution of cerebral functions; nevertheless, the onset of associated disorders was, perhaps, the price to be paid for this benefit [16].

Microsatellites in Tumors: Possible Genetic Markers?

A genetic marker, by definition, must more have two or more alleles. When the frequency of the most common allele is smaller than 95%, the marker is considered polymorphic; the degree of polymorphism of genetic markers is measured by the PIC, acronym of "polymorphism information content", which may range from 0,0 to 1,0.

For each specific marker, the PIC is computed according to the number of different alleles related to such markers and according to the frequency of the appearance of such alleles in a given population. The ideal genetic map is made up by the high density of markers, as well as by a PIC of at least 0,7 [30].

Summing up the data reported above, the following are the microsatellite characteristics [2, 3]:

1) Microsatellites are polymorphic, i.e. a microsatellite may have different allelic forms; alleles of a given microsatellite have the same repetition unit, and the polymorphism depends only on the total length of the allele, i.e. on the total number of repetitions of the same base unit. Each individual has two alleles for each microsatellite, one of paternal origin and one of maternal origin: if they are the same, each individual will be a homozygote for that specific microsatellite, otherwise he will be a heterozygote.

2) The inheritance of the single alleles is assessed by means of PCR reactions, carried out by using primers designed to join to flanking sequences, i.e. the ones next to the microsatellite target.

Amplification products may be visualized by means of a common electrophoretic run on poliacrylammide gel, by using bromide ethydium or silver staining. Since the allelic polymorphism is generally made up by differences of a few base pairs, if not of a single base pair, the discriminating ability of the poliacrylammide is not enough to detect such small differences [26, 27].

This makes alternative survey methods, with a proven resolution power, necessary. The solution might consist in using sequencing gels, that enable also the separation of products that differ by a single nucleotide. At present, there are also more complex methods which ensure a definitive solution to the issue of highlighting polymorphism.

Such techniques are based on semi-automatic fluorescence methods, in which the primers used in order to amplify the microsatellites are marked covalently with various fluorochromes. The system includes known weight standards to assess the size of amplification products, which are also marked by fluorochromes different from those used for the primers [26, 27].

The aforesaid amplification products and the internal standards are "pre-mixed" together, and are made to run in the same capillary electrophoresis. As a result, it is possible to analyze more than one microsatellite and to compute, at a time, its size, by means of a computerized system that compares sets of markers with known weight.

Therefore, this system contributes in discriminating a microsatellite from another on the basis of the wavelength emitted from the fluorochrome, promoting estimates of the size of products by comparing them with DNA fragments of known size. This technique does not, however, contribute to revealing the nucleotide sequence of alleles, that can appear only by means of sequencing techniques [16].

Tumour Study Approach for Studies by Analyzing the Candidate Gene

The candidate gene approach consists in selecting for the study a specific gene, considered to be the most probable site of mutation [2, 3].

The main gene selection criteria being the following:

1. the genes are selected because they appear to be defective in similar genetic disorders, albeit in other animal species (generally in rats);

2. the genes are selected according to their function, and if they are likely to be involved in the disease.

The analysis of the candidate gene consists in sequencing the entire gene by comparing a group of healthy individuals with the patients. Nevertheless, the presence of a mutation in a gene is not sufficient to identify the cause of the disorder, and many differences in the sequences are only polymorphisms.

Should the change in the DNA sequence cause an amino acidic substitution in an important portion of the corresponding protein, it would be considered as a "*strong*" candidate [26, 27].

The genetic modification responsible for the disorder, will co-isolate with the disease; this can be assessed by analyzing entire families, if available, in order to verify if the inheritance of the mutation is associated to the inheritance of the disease.

Unfortunately, for many genetic diseases the relevant candidate gene has not been detected. Moreover, various hereditary diseases, similar between different species, can derive from mutations on completely different genes [29, 30].

In conclusion, the approach with candidate genes has the advantage of enabling the identification of the specific mutation and, consequently, of carrying out aimed genetic tests. By the same token, it is an expensive method as it requires the sequencing of the entire gene, that might turn out to be unchanged.

Linkage Analysis and Microsatellites in the Study of Neoplasms

The method of "*linkage analysis*" is based on totally different pillars compared to the candidate genes approach, the main difference consisting in the fact that in the former, no assumption is made on which gene is accountable for disease, nor, more in general terms, which chromosome trait is involved.

In this method the entire genome map is potentially the subject of the analysis, with no special attention to a specific region [26, 27].

The concept on which the theory of the linkage analysis is based, consists in the fact that, during the meiotic event that leads to gamete formation, homologous chromosome pairs approach one another exchanging genetic material (a.k.a. crossing-over). DNA regions mapping close to that same chromosome tend to be co-inherited during the meiotic event; the closer these regions are, more probabilities will there be that they are inherited from one generation to another. [26, 27].

At the basis of the linkage analysis, there must be a family (sick individuals and carriers) with at least 3 generations, in order to make an estimate of traits segregation from one generation to another. Chromosome localization of the mutation responsible for the disease may be performed by using genetic markers, i.e. variable DNA regions within a population, with a known chromosome position. The more such markers are found near the mutation site, the more will they be likely to be co-inherited, together with the mutation from a parental generation to another. Thus, the genetic marker will act as a "guide" for the chromosome localization of the locus of the disease.

To fully comprehend the theory of linkage analysis, it is necessary to underline that the markers to be employed in the study must necessarily be variable, i.e. with allelic variations among the population. In fact, if a marker were not polymorphic, and were to be found being the same across the population range, it would be impossible to identify the disease site, not even if it were associated to it, because it would be the same in the healthy subject and in the ill one.

Consequently, for a linkage analysis to be successful, the following indispensible conditions must occur:

- the marker must be physically near the locus of disease;
- the marker must be polymorphic within the population and must have a rare allelic variant, which appears only in association with the disease.

Markers normally employed to carry out this type of study are microsatellites. These are considered as the ideal genetic markers because they are abundantly scattered across the genome and are highly polymorphic [16].

The number of microsatellites used to perform a linkage analysis is not established; it is generally higher the greater the probabilities for the study are to be successful. This assumption derives from the fact that, not addressing the attention towards one given gene or chromosome portion in particular, the genome screening should be the broadest, containing the highest number of markers, across the entire genome.

Usually, this type of investigations are widely applied in studies of most animal species. For example, in order to carry out a linkage study within a family tree, 200-300 microsatellites are used, in pedigrees including at least one hundred animals [26, 27].

For a given genome area, the probability that a recombination event occurs between a marker and a gene of the disease, is directly proportional to their distance; the probability that such event occurs is known as "recombination fraction". In fact, if it is equal to 0.5, the marker and the gene of the disease "are not linked" and are, therefore, independently segregated. Conversely, if a marker and the disease gene are linked, the "recombination fraction" is lower than 0,5. The linkage analysis is therefore a more complex study and does not represent the end stage of the investigation. Such analyses, in fact, lead to characterizing a chromosome region which might be the locus of the disease.

A further linkage analysis is consequently performed, using the highest number of microsatellites present in the region, in order to focus down on the chromosome portion mapping the genetic disorder.

Subsequently, the analysis will follow the candidate genes approach, i.e. detecting all genes mapping that region, and performing a sequence analysis comparing healthy individuals (wild-type) with sick ones (mutant-type).

Although the linkage analysis is a complex approach, it is the only way out when a priori assumptions cannot be made on candidate genes, being the pathways and processes involved in the disease unknown [2, 3].

Candidate-Linkage Approach and the Use of Microsatellites

There is a further genetic approach in the study of tumoral diseases, which is a kind of synthesis between the candidate gene method and the linkage analysis.

As for the linkage analysis, also this method envisages the employment of microsatellites.

However, conversely from the linkage analysis, the choice of microsatellites to be analyzed is completely different.

In linkage analysis, in fact, a high number of microsatellites is analyzed, possibly randomly distributed throughout the genome. The "*candidate-linkage*" is a specifically aimed linkage, as the analyzed microsatellites are either near or inside the intronic sequences of candidate genes [26, 27].

The "*candidate-linkage*", consequently, represents a less expensive strategy in terms of time and costs, if compared to the candidate genes approach, which envisages the sequencing of entire genes, or compared to the traditional linkage, because it contributes to investigating on precise genomic regions including genes that could be been involved in the disease. However, for this method to lead to factual results, the following conditions must take place:

- the candidate gene selected must contain one or more microsatellites;
- such microsatellites must be polymorphic;
- in case the candidate gene is responsible for the disease, the microsatellite must have a rare allelic variant, appearing only in combination with the disease.

In conclusion, there is no absolutely best method in the study of a genetic disease [3].

Microsatellites and Endometrial Cancer

Endometrial cancer studies take into account microsatellites already analyzed in HNPCC target genes, located in proximity of or within the intronic sequences, as shown on Table **3** [4, 31-36].

For such genetic loci a candidate-linkage analysis has already been carried out, in order to characterize the associated cancer markers: the phenotypic consequences of mutations in "*MisMatch Repair*" (MMR) genes are represented by the MSI and the LOH.

Although MSI is an independent predictor of a favourable outcome in colon-rectal cancer, its prognostic association in endometrial carcinoma is controversial [3, 14].

In fact, in author' current researches, the MSI investigations are developed by Genequality Kit for CC-MSI, n 0461 (AB Analitica srl, Padua, Italy, www.abanalitica.it), specific for colon-rectal cancer detection.

In scientific literature, some studies reported the association of MSI with poor prognosis, other studies founded an association of MSI with a more favourable outcome, and other reports reported MSI to be of no prognostic consequence.

Moreover, the prognostic significance of MSI in high grade endometrial cancer needs to be still defined [14].

This changed phenotype reflects the inability to repair small insertions or nucleotide deletions, caused by non-corrected DNA replication, and leads to a new allelic somatic tissue, confirming the elevated degree of genetic disorder.

Many studies have evaluated the MSI in endometrial cancer specimens, comparing electropherograms of neoplasms with healthy tissues in the same subject, allowing to estimate if such genetic loci, detected in the colon cancer, were unstable also in the endometrial cancer, considered the close correlation between the two neoplasms.

Comparisons of the electropherograms of healthy tissue, of tumoral tissue, and in the peritoneal washing, would reveal any genetic changing of cells taking place in the peritoneal area.

The instabilities of the mono and dinucleotide molecular markers of "short tandem repeats" (STR) have been found in a series of malignant neoplasms in the cutis, mammalian glands and in the colon, apart from the endometrium.

According to the type of malignant neoplasms observed, results of molecular biological analysis performed by various STRs, demonstrated instability of the markers. This phenomenon may be observed in electropherograms, as allelic increase and/or LOH [3, 14].

It is not a simple task to identify which genes have been directly involved in such pathologies. It is, in fact, clearly difficult due to the presence of numerous genes next to the loci studied.

There is no preselected panel of microsatellite markers, since many derive from the broader traditional Bethesda Panel (BAT25, BAT26, D2S123, D5S346, D17S250) and the introduction of mononucleotide markers, dinucleotide markers and two tetranucleotide markers.

In 1995, TGFβRII was thought to be the first targeted gene in colorectal cancers, with a high MSI [30]. In such gene, the microsatellite sequence of poli $(A)_{10}$ is located at the beginning of the codifying sequence, which implies that frameshift mutations unavoidably lead to the inactivation of such oncosuppressor genes, because of the loss of the normal reading frame [37].

The analysis of several tissues at a time,by Duval *et* al, has lead to the observation of how the endometrial tissue is less affected by MSI since, compared to colorectal and gastric tumors, the frequency of mutations is undoubtedly lower both in codifying regions and in intronic sites [37].

Table 3. Investigation of MMR Genes Responsible for Microsatellite Instability in Patients with Endometrial Cancer

MMR Gene Studied	No. Patients	Proportion of Tumors With Alterations	Genetic Approach	References
MLH1	18	22% (4/18)	– polymerase chain reaction amplification – denaturing polyacrylamide gel electrophoresis	[4]
MLH1, MSH2	30	23% (7/30)	– polymerase chain reaction amplification – polyacrylamide gel electrophoresis	[31]
MLH1	14	86% (12/14)	Methylation studies (MLH1 promoter)	[32]
MSH2		14% (2/14)		
MLH1	12	25% (3/12)	MLH1 and MSH2 protein expression	[33]
MSH2		25% (3/12)		
MLH1, MSH2, MSH6	58	8.6% (5/58)	–Sequencing (MLH1, MSH2, MSH6) –MLPA (MLH1, MSH2)	[34]
MLH1, MSH2, MSH6	81	4.9% (4/81)	–Sequencing (MLH1, MSH2, MSH6) –MLPA (MLH1, MSH2, MSH6, PMS2) –Methylation studies (MLH1 promoter)	[35]
MLH1, MSH2, MSH6	100	9% (9/100)	Sequencing (MLH1, MSH2, MSH6) Large deletion testing (MLH1, MSH2, MSH6)	[36]

Abbreviations: *MLPA: Multiplex Ligation-dependent Probe Assay.*

Consequently, there are marked qualitative and quantitative differences in various tumors MSI-H, suggesting that in such tumors the profile of mutations in target genes is definitely tissue-specific [37].

The TGFβRII does not appear to be particularly unstable, as confirmed in literature by in Myeroff, who found 92% of instability (MI+) in the colorectal cancer and 81% in stomach cancer, but only 19% in endometrial tumors [38].

The *TGFβRII* gene is altered in the majority of MI+ colorectal and gastric carcinomas, in 90% and in 71% respectively, but Gurin *et al.* failed to identify any MI+ endometrial cancer with these alterations. A previous study reported a low incidence of *TGFβRII* mutations in MI+ endometrial cancer compared to MI+ colorectal cancers. Together, these findings indicate that mutational inactivation of *TGFβRII* do not provide any significant contributions to the tumorigenic process in cellular endometrial pathways [39].

Therefore, MSI appears as an unstable marker, depending on the tissue analyzed; in endometrial tumoral tissue it generally turns out to be rather stable.

These markers can be located either in intronic regions or in codifying regions. In the endometrial somatic tissue, in fact, four genes seem to be involved in carcinogenesis, namely: BAX, hMSH3, hMSH6 and IGFIIR. They are also characterized by instability in codifying sequences, with mononucleotide repetitions. Conversely, other studies show that mononucleotide repetitions in intronic sequences are mutated in approximately 50% of colorectal tumors [40].

Today, there is still no clear explanation of such instability in non-codifying regions. The more probable hypothesis is that such instability might involve splicing donor or receiver sites, and be, consequently responsible for alternative "splicing" phenomena, with consequent alteration or loss of functionality of the respective proteins.

An interesting aspect, confirmed by Duval *et al.* [37] as well as by other authors [3, 14], is that mononucleotide markers are among the most unstable markers, as observed also in this review for NR21, NR24 and BAT 40. Mononucleotide markers BAT25 and BAT26 represent an exception in endometrial tumoral tissue, since not all patients show a stable profile.

The locus BAT25 contains a 25 timine trait, localized in intron 16 of the proto-oncogene c-Kit, located on 4q12. Such gene codifies for receptor-protein KIT, involved in the transduction of cellular proliferative signals. As a result of mutations in the gene, the receptor can be activated without control, inducing the cell to divide without the right stimulation. Locus BAT26 contains a 26 adenine trait, located on intron 5 of human MutS homologue (hMSH2), an oncosuppressor gene involved in the repair of the mismatch during DNA replication, located on site 2p22-21. Microsatellites NR21 and NR24 are sequences of 21 and 24 timine, respectively located in the 14q11.2 and 2q11.2 chromosomic regions.

These two markers are added to the panel due to their high sensitivity and specificity [40], albeit being remarkably unstable, as confirmed in literature, especially colon cancers.

Marker NR21, in particular, is particularly interesting since it stands in a locus where gene TSG14C is located; the TSG14C is an oncosuppressor gene involved in the squamous cells carcinoma of the esophagus, in the nasal-pharyngeous carcinoma and the meningioma.

Mononucleotide marker BAT40 is characterized by 40 adenine and is located in the 1p12-13.3 chromosomic region. It has been selected for its instability association with uterus

cancer, one of the most frequent HNPCC-associated tumors. BAT40 has proved to be particularly unstable in the endometrial tissue. An interesting article by Samowitz *et* al [41] highlights how markers BAT26 and BAT40 have been used to analyze the colon cancers. The author pinpointed a remarkable instability of both markers, in that tissue, as expected, and proved that BAT40 is much more unstable in sporadic carcinomas (26,6%) than in colon adenomas (2,5%).

This means that the instability is a relatively late event in such tissue, considering the typical adenoma/carcinoma sequence [41].

The GSTM1 is the potential gene mapping BAT40, a glutatione-S transferase. This oncosuppressor is one of the main targets in endometrial cancer. Dinucleotide marker D17S250, mapping in locus 17q12, seems unstable in various tumors, ranging from HNPCC to familial or sporadic breast cancers. Among the major potential candidate genes located on such loci, the main role is played by the thyroid hormone receptor α (TRα) and by the retinoic acid receptor α (RARα) [42].

According to its normal cell functions, also the gene TBC1D3, located on locus 17q12, seems to be involved since it codifies for an oncoprotein activating RAS and modulating the epidermal growth factor receptor (EGFR); moreover, such gene is amplified in 15% of prostate cancers [43].

Also the gene GRB7 located on same locus, pertains to the growth factor receptor bound protein family (GRB), and has a clear involvement due to its key function in the cell growth.

D17S250 has been seen to be unstable also in familial and sporadic breast carcinoma [42], in prostate cancer [44] and in ovarian cancer [45].

The dinucleotide marker D18S58, added to the traditional Bethesda Panel, is located in the telomeric region of chromosome 18, nest to the "deleted colorectal cancer gene" (DCC), an oncosuppressor that codifies for a protein of cellular adhesion, which seems to be absent or barely expressed in the late stage of colorectal cancer.

Two more dinucleotide markers, present in the Bethesda Panel, are D2S123 and D5S346.

They seem to be unstable not only in endometrial tissue, but also in other tumoral tissues. D2S123, for example, is unstable also in ovarian cancers [46] and in stomach cancers [47], while D5S346 seems to be unstable in ovarian cancer [48] and subject to loss of heterozigosity in large-cell neuroendocrine lung carcinoma [49].

CONCLUSIONS

The scientific evidence submitted in this review, shows the possibility of using these new genetic biomarkers -MSI and LOH- in the endometrial mucosa.

In cellular endometrial pathways, they can both be associated with the loss of cell control and, therefore, to the apoptosis leak, with a consequent onset of tumors in the endometrial tissue. Hence, due to the high incidence of endometrial tumors associated with colon cancers, broadly discussed in literature, the study of endometrial and colorectal tumors might be performed by using the same genetic markers, highlighting the possibility to anticipate a neoplastic transformation in both tissues. The study of genetic molecular markers, such as MSI, represents an attractive alternative that might potentially help in constructing better risk-stratification models to direct adjuvant therapies and promote the development of novel targeted treatments.

This method may, therefore, enable the assessment of the state of cellular replication, by means of an MSI evaluation in the loci examined, thus anticipating an alteration in the mismatch repair system, entailing the control of the entire cell replication process, with important fallout on clinical prevention programs.

CONFLICT OF INTEREST STATEMENT

Authors certify that there is no present or potential conflict of interest in relation to this article and they reveal any financial interests or connections, direct or indirect, or other situations that might raise the question of bias in the work reported or the conclusions, implications, or opinions stated – including: employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/ registrations, and grants or other funding for the individual author(s) or for the associated department(s) or organization(s), personal relationships, or direct academic competition.

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