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Double strand break repair components are frequent targets of microsatellite instability in endometrial cancer

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ABSTRACT

Aim: DNA double strand break (DSB) repair is a central cellular mechanism of the DNA damage response to maintain genomic stability. DSB components are frequently mutated in colorectal cancer with microsatellite instability (MSI). We investigated whether DSB repair is involved in endometrial cancer (EC) with MSI.

Methods: Mononucleotide microsatellite tracts of 14 genes of the DSB repair system were analysed in a series of 41 EC with MSI. Among these genes, the *microcephalin 1* (*MCPH1/BRIT1*) has never been tested as target of MSI in tumour series.

Results: The most frequently mutated gene was *DNAPKcs* ($n = 14$, 34%) followed by *RAD50* ($n = 7$, 17%), *MRE11*, *ATR* and *BRCA1* ($n = 6$, 15%), and by *CtIP* and *MCPH1* ($n = 5$, 12%). While DSB biallelic mutations were infrequent, a high proportion of tumours ($n = 30$, 73%) presented mutations at some component of the DSB repair pathway, and almost half of them showed alterations at two or more components. Tumours with mutations in two or more genes were significantly associated with advanced grade ($p = 0.03$) and vascular invasion ($p = 0.02$) and marginally associated with advanced stage ($p = 0.07$).

Conclusions: Our results suggest that in EC, the DSB repair is a relatively common mutational target of MSI and might contribute to tumour progression, and also that *MCHP1* may be a novel target gene of MSI.

1. Introduction

Microsatellite instability (MSI) is the hallmark of cancer with DNA mismatch repair (MMR) deficiency, and a widespread phenomenon throughout the genome. It consists in the accumulation of hundreds of thousands of somatic insertions/deletion mutations in simple repeated sequences known as microsatellites.[1] Among this bulk of alterations, cancer arises

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when mutations occur at cancer genes, such as those involved in cell growth and survival and DNA repair,. especially in short microsatellite repeats in coding regions present in target genes.[1,2] MSI is the main diagnostic feature of the majority of hereditary non-polyposis colorectal cancer (HNPCC), and also of a subset of sporadic colorectal, gastric and endometrial tumours.[2,3] In endometrial cancer (EC), MSI is present in about 20% of tumours and, as in the majority of sporadic tumours, is mainly caused by epigenetic silencing of the MMR gene *MLH1*.[4]

The DNA damage response (DDR) consists of intricate signalling networks that detect DNA lesions, signal their presence and promote their repair. These sensory networks impact on countless cellular functions (cell cycle, apoptosis, chromatin structure, etc.) and orchestrate cell regulation at multiple levels. DDR preserves genomic stability, and defects in the ability to properly respond to and repair DNA damage increase the risk of tumour development. Several mechanisms are known to participate in the DDR, such as MMR system, double strand break (DSB) repair, nucleotide excision repair (NER), base excision repair (BER), trans-lesion bypass mechanisms, direct lesion reversal and Fanconi anaemia pathway.[5] In the last years a great progress has been made towards understanding the DDR, although studies about how the different participants interact and are controlled are constantly being released.

Cells use two main mechanisms to repair DSBs, non-homologous end-joining (NHEJ) and homologous recombination (HR), depending on the phase of the cycle and the nature of the DSB ends. NHEJ can occur throughout the cell cycle and is the major pathway for the repair of DSBs in multicellular eukaryotes, whereas HR is restricted to late S or G2 since it uses sister-chromatid sequences as a template to repair.[5] DSB repair components are potential targets of MSI. In colorectal cancer (CC), Miquel et al.[6] have recently studied many genes of this DNA repair network. The authors found that a high proportion of tumours carried alterations at one or more components of NEHJ and HR pathways. Such an exhaustive analysis has not been performed so far in EC with MSI, although mutations in several genes have been previously reported.[7–9]

In the present study we investigated 14 DSB repair genes as possible targets of MSI in EC. We also estimated the association between mutations and clinicopathological variables. Among the 14 candidate genes analysed, we included *microcephalin 1* (*MCPH1/BRIT1*), a novel candidate tumour suppressor gene, that has never been tested as target of MSI in tumour series. Although the gene was initially identified as involved in determining the brain size, recent studies suggest that MCPH1 participates in essential biological processes such as the regulation of mitotic entry, the maintenance of genomic stability and the DSB repair.[10–12] Besides, reduced expression of MCPH1 has been reported in ovarian, prostate and breast tumours.[11]

2. Material and methods

2.1. Tumour samples and MSI classification

Our series consisted of 41 EC patients with MSI from a previously described series.[13] The study was approved by the institutional ethical committee and all subjects provided informed consent. MSI determination and classification were performed by using five recommended quasimonomorphic mononucleotide markers (BAT25, BAT26, NR21, NR24 and NR27) according to published conditions and criteria, with some modifications.[14] Briefly, primer sequences of markers were as reported and were amplified in pentaplex in 25 μL reaction volume containing 20 ng of DNA, 240 nM of each primer pair for NR21, NR24, NR27 and BAT25 and 1 μM of each primer pair for BAT26, 200 μM of deoxynucleotide triphosphates, 1× commercial Biotaq Reaction Buffer, 2 mM $MgCl_2$ and 1 U Biotaq DNA Polymerase (Bioline). Thermocycling conditions were: 94 °C, 5 min, followed by 12 cycles of 30 s at 94 °C, 30 s at 72 °C 30 s and 30 s at an annealing temperature that decreased 2 °C every 3 cycles (beginning at 61 °C in the first 3 cycles and decreasing to 55 °C in the last 3 cycles) and 25 cycles of 30 s at 94 °C for 30 s, 30 s at 72 °C and 30 s and 55 °C. A final extension step was carried out at 72 °C for 10 min, followed by storage at 4 °C. The PCR products were diluted 1:50 in distilled water and 1 μL of the dilution was mixed with 9.75 μL of formamide and 0.25 μL of GeneScan 500 LIZ Size Standard (Applied Biosystems), denatured at 98 °C for 5 min and cooled on ice. Fragments were separated by automated capillary electrophoresis in an ABI Prism 3100 16-capillary genetic analyser (Applied Biosystems) and electropherograms were analysed using the Peak Scanner Software v1.0 (Applied Biosystems).

2.2. Frameshift mutation detection in genes of the DSB repair

Frameshift mutations within mononucleotide tracts of 14 genes involved in the DSB repair were analysed. Regions encompassing mononucleotide repeated sequences of genes were amplified in monoplex PCR reactions. For each reaction 20 ng of genomic DNA, 240 nM of each forward and reverse primer, 200 μM of deoxynucleotide triphosphates, 1× commercial Biotaq Reaction Buffer, 2 mM $MgCl_2$ and 1 U Biotaq DNA Polymerase (Bioline) were mixed in a final volume of 25 μL. The forward primer of each pair was marked with the fluorescent dye 6-FAM. Thermocycling conditions were: 94 °C, 5 min, followed by 35 cycles of 30 s at 94 °C, 30 s at the corresponding annealing temperature, and 30 s at 72 °C, with a final cycle of extension for 10 min at 72 °C. Details of PCR conditions for *MCPH1* analysis are shown in Table 1. For the other genes additional information is available upon request. Different groups of amplicons were mixed for simultaneous genotyping. Thus, 1 μL of each amplicon was mixed and diluted up to 50 μL in distilled water, 1 μL of the dilution was mixed with formamide and the size standard, and analysed as explained above. Deletions or insertions in the repeated tracts were identified based on peak pattern alterations when compared to normal DNA sample profiles. Fig. 1 illustrates some electrophoretic profiles of *MCPH1* mononucleotide repeats.

2.3. Statistical analysis

The χ-squared and Fisher's exact non-parametric tests were used to analyse the relationship between the number of mutated DSB components in each tumour and the clinicopatho-

Table 1 – PCR details used for the analysis of *MCPH1* mononucleotide repeats.

Primers	Repeat	Exon	Product size (bp)
F: 5′-TATACAGATGCAGGACAGCTG-3′ R: 5′-TGCCATTATCTACATTGAAATC-3′	A_9	4	127
F: 5′-AAATGTATGCAGCCCAAAGA-3′ R: 5′-GGACACAAATGCAAAGAACTG-3′	A_6	5	153
F: 5′-TCTAAGAAGGAGAGAACAAGC-3′ R: 5′-GACTGGAGATGGTTTTTGCTG-3′	A_7	8	109

F, forward primer; R, reverse primer; bp, base pairs.
Annealing temperature: 61 °C.

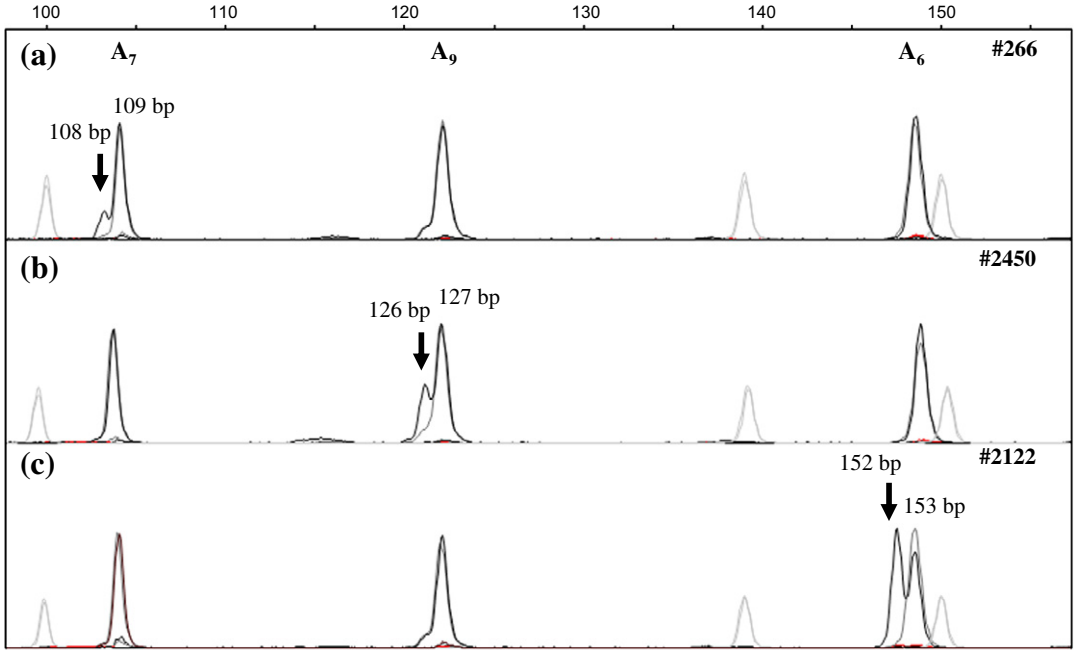

Fig. 1 – Examples of tumours with frameshift mutations in coding mononucleotide repeats of *MCPH1*. Mononucleotide deletions are indicated by arrows in the A_7 (a), A_9 (b) and A_6 (c) tracts of exons 8, 4 and 5, respectively. Black, tumour; grey, control; light grey, size standard; bp, base pairs. Tumour reference code is indicated.

logical variables. All analyses were two sided, and significance was set at a *p*-value of 0.05. Analyses were performed using GraphPad Prism statistical package (v. 5).

3. Results

The incidence of mutations at mononucleotide repeats of genes involved in the DSB repair is shown in Fig. 2. The most frequently mutated gene was *DNAPKcs* (also known as *PRKDC*), displaying mutations in 34% (14/41) of tumours, followed by *RAD50* (17%). *MRE11*, *ATR* and *BRCA1* were mutated in 15% of tumours, and *CtIP* (*RBBP8*) and *MCPH1* in 12%. The rest of the genes (*CHK1*, *XRCC2*, *WRN*, *CtIP*, *CDC25C*, *BLM* and *BRCA2*) were mutated at a lower rate (0–7%). Among the *MRE11* alterations, since the T_{11} mononucleotide tract is non-coding, only those that are known to significantly impair wild-type protein expression were scored (see Section 4). Regarding the mutations found in *MCPH1* gene, two tumours presented deletions of one nucleotide in the A_9 tract of exon 4, other within the A_6 microsatellite tract of exon 5, and two more tumours carried single nucleotide deletions within the A_7 run at exon 8 (Fig. 1). This gene was also analysed in 62 microsatellite stable (MSS) EC and no mutations were found (data not shown).

Mutations at any of the DSB repair genes analysed were found in 71% of the tumours (29/41), and almost half of them (14/29, 48%) presented alterations at two or more components of the pathway. Of the 29 tumours with mutations, NHEJ and HR components were found mutated in 14 (48%) and 24 (83%) tumours, respectively.

When tumours were categorised according to clinicopathological characteristics of patients, tumours showing mutations in two or more genes of the DSB repair system were significantly associated with advanced grade ($p = 0.03$) and vascular invasion ($p = 0.02$) and marginally associated with advanced surgical stage ($p = 0.07$) (Table 2).

n° of cases	tumor code	DNAPKcs	RAD50	MRE11	ATR	BRCA1	CtIP	MCPH1	CHK1	XRCC2	WRN	TOPBP1	CDC25C	BLM	BRCA2	n° of mutated genes
		A10	A9	T11	A10	A8	A9	A6,A7,A9	A9	T8	A8	A8	A8	A9	A8,A8	
1	266			-1-2	+1			-1	+1	-1						5
2	835	-1	-1	-1-2		-1										4
3	2356	+1	+1	-1-1		-1										4
4	1971	-1-1	-1-1				+1	-1								4
5	2450		-1				-1	-1		-1						4
6	2293	+1	+1	+1										-1		3
7	1513	-1		-2					-1							3
8	2297	-1		-1-1	+2											3
9	294				+1						-1	-1				3
10	1000	+1		-2												2
11	1357			-1	-1	-1										2
12	1936	-1		-1		-1										2
13	1128					-1	-1									2
14	744	-1							-1							2
15	1346	-1		-1												1
16	518	-1														1
17	2113	-1		-1												1
18	1647	+1														1
19	1802	-1														1
20	1731		-1	-1												1
21	1861		-1	-1												1
22	2422				-1											1
23	1001				-1											1
24	1911					-1										1
25	2035			-1			-1									1
26	2111						-1									1
27	1401			-1				-1								1
28	2122			-1				-1								1
29	1440										-1					1
30	863												+1			1
31	1740															0
32	2098			+1												0
33	2189															0
34	2041															0
35	738			-1												0
36	1389			-1												0
37	1398			-1												0
38	1640			-1												0
39	1658															0
40	2445															0
41	2480															0
		14/41	7/41	6/41	6/41	6/41	5/41	5/41	3/41	2/41	2/41	1/41	1/41	1/41	0/41	
		34%	17%	15%	15%	15%	12%	12%	7%	5%	5%	2%	2%	2%	0%	

Fig. 2 – Frameshift mutations in mononucleotide tracts of DSB repair genes in MSI endometrial tumours. Wild-type, white box; mutant, black box; mutation in *MRE11* with little or unknown effect on the expression of wild-type protein, grey box. −1, 1 bp deletion; −2, 2 bp deletion; +1, 1 bp insertion; +2, 2 bp insertion. Cases with homozygous (−1, −1) deletions cannot be distinguished from hemizygous mutations of one nucleotide deletion and LOH of the other allele.

4. Discussion

An important number of tumours of our series showed mutations in *DNAPKcs*. The mutation incidence that we observed (34%) was significantly higher compared to what has been described in EC with MSI (13%, $p = 0.02$; number of cases (n) = 54).[7,15] Similarly, the rate of alterations detected in *RAD50* (17%) was significantly superior than the one reported for MSI EC (2.4%, $p < 0.01$; $n = 42$).[7] Finally, we found that BRCA1 was mutated in 15% of cases which contrast to the absence of mutations found among 64 MSI EC accounted in the literature.[8,16,17] The observed discrepancies could be due to

Table 2 – Relationship between tumours, classified by the number of mutated DSB components and clinicopathological characteristics of patients.

Characteristic	(*n*)	Number of mutated genes		*p*-Value[b]
		0–1	⩾2[a]	
Age at diagnosis				
<Median (67 years)	(20)	13	7 (35)	1.00
⩾Median	(21)	14	7 (33)	
Stage				
I	(22)	17	5 (23)	0.07
II	(10)	6	4 (40)	
III	(9)	4	5 (56)	
Grade				
1	(14)	12	2 (14)	0.03
2	(12)	8	4 (33)	
3	(15)	7	8 (53)	
Myometrial invasion				
0–<50%	(30)	21	9 (30)	0.46
>50%	(11)	6	5 (45)	
Vascular invasion				
No	(25)	20	5 (20)	0.02
Yes	(16)	7	9 (56)	

[a] Number (percentage).
[b] Calculated by χ-squared (for trend) or Fischer' exact tests.

the low number of cases analysed together with the false negatives due to the lack of sensitivity in detection methods. *TOPBP1* and *XRCC2* genes have not been previously studied in EC, and, as in CC,[6] the observed mutation rate was very low.

It is known that not all the mutations at the non-coding T_{11} tract of *MRE11* gene have a functional impact. Thus, monoallelic deletions of two or more nucleotides have been reported to dramatically reduce the amount of MRE11 protein, whereas only slight or no reduction has been observed when only one nucleotide is deleted.[18–20] In addition, biallelic deletions of 1 bp have been associated to a strong impairment of MRE11 protein expression in tumours and also to a reduced functionality in cell line assays.[19–22] Therefore, only biallelic mutations or deletions of two or more nucleotides were considered (Fig. 1). We do not have any information regarding whether or not 1 bp insertions are deleterious.

To date, mononucleotide coding tracts of *MCPH1* gene have not been analysed in MMR-deficient tumours. Twelve per cent of tumours in our series carried deletions in the studied repeated sequences of this gene. Two tumours presented deletions of one nucleotide in the A_9 run of exon 4 and other within the A_6 repeat of exon 5. In either case the mutations are predicted to generate a truncated, and presumably non-functional, protein of 145 aa. A homozygous germline insertion of an A at the A_6 tract of exon 5 has been described in two brothers with a primary microcephaly syndrome known as PCC (postnatal short stature and premature chromosome condensation).[23] The resulting frameshift was predicted to generate a termination codon in exon 6 which would result in a markedly truncated protein of 146 aa, encoding only the N-terminal BRCT domain of microcephalin.[23] Two more tumours of our series presented single nucleotide deletions within the A_7 run of exon 8 that would produce a truncated protein of 499 aa. Since the entire protein consists of 835 aa, almost half of the protein would be lost and the resulting protein would lack the two C terminus BRCT domains that are important for mediating DDR.[24] In breast cancer these two domains were found deleted due to a 38 base pair deletion in exon 10 that resulted in a non-functional truncated protein of 656 aa.[11] Accordingly, the alterations we found generate non-functional proteins. Considering that mutations in *MPCH1* gene were observed in MSI, but not in MMR-proficient tumours, we propose that this gene is a target gene of MSI in EC.

In agreement to the features of MSI tumours,[25] most of the mutations that we observed were monoallelic. However, it has been reported that heterozygous mutations at some components of the pathway may decrease the overall DNA DSB repair activity in cells.[21,26,27] An example is DNAPKcs which plays a central role in NHEJ repair leading to the recruitment and activation of end-processing enzymes, polymerases and DNA ligase IV.[28] A subclone of HCT-8 cell line carrying a heterozygous deletion at the A10 repeat within the exon 5 of DNAPKcs gene has been shown to be more sensitive to a combined treatment of bleomycin plus an enzyme inhibitor, compared to the original wild-type HCT-8 cell, suggesting that inactivation of one of the two alleles of the gene decreases the overall DNA DSB repair activity in the cells.[21] The HR signalling pathway has also been described as unusually sensitive to haploinsufficiency. For instance, it has been shown that heterozygous alterations in *ATR*, *RPA1* or *RFC2* confer an impaired ability to respond to DNA damage.[26,27] Besides, monoallelic *ATR* mutations in the A_{10} repeat of exon 10 have been associated with poor clinical outcomes in endometrioid EC with MSI.[9] Therefore, it is tempting to suggest that tumours with haploinsufficiency at specific DSB components may acquire survival advantage and contribute to tumour progression in EC with MSI.

It could be questioned whether mutations at genes showing low mutation frequency contribute or not to DSB repair impairment. However, according to the accumulative haploinsufficiency model proposed for tumours with MSI, monoallelic alteration of several genes whose products participate in the same molecular network could also result in impairment of the pathway.[25,29] In this regard, almost half of the mutated tumours in our series showed mutations in more than one component of the DSB repair pathway. Furthermore, the tumours carrying two or more mutated genes also appeared to display different behaviour since they were associated with higher grade and vascular invasion and showed a tendency to advanced stage. Moreover, the relationship between the simultaneous alteration of two or more genes of the DSB repair pathway and clinicopathological variables remained unaltered when we disregarded those genes with a mutation incidence below to 8%, the cutoff value suggested to discriminate real target from bystander genes in EC with MSI by some authors (not shown).[7]

Impairment of DSB mechanisms has been considered an important topic in cancer treatment since it would be expected that cells with DSB deficiency may display different sensitivities to certain chemotherapy drugs or to ionising radiation. In fact, *in vitro* studies have reported that cells with

mutated components of the DSB repair show increased sensitivity to chemotherapeutic agents (including those generating DSBs).[21,22,30] On the other hand, we found that the presence of MSI may predict a worse response to radiotherapy of early-stage endometrioid EC patients,[31] and similar results were found almost at the same time in rectal cancer with MSI.[32] In addition, it has been reported that the loss of distal 11q chromosome including three critical genes involved in DSB repair (*MRE11*, *ATM* and *H2AFX*) was associated with a reduced sensitivity to ionising radiation in head and neck squamous cell carcinoma.[33] Recent findings have linked MLH1 to DNA repair recombinative processes, independently of its role in MMR. In particular, after induction of DNA DSBs, MLH1 has been shown to participate in preventing error prone HR processes, showing that the absence of MLH1 could increase the mutator phenotype of cells with MSI by also affecting the DSB repair system in addition to the MMR system.[34,35]

In summary, our results suggest that coding mononucleotide tracks of DSB repair genes are common targets of MSI in EC, and that the accumulation of monoallelic alterations in multiple genes of this pathway might contribute to a worse clinicopathological condition.

Conflict of interest statement

None declared.

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