Rev Port Pneumol. 2014;**20(1)**:20–30

revista portuguesa de
PNEUMOLOGIA
portuguese journal of pulmonology
www.revportpneumol.org

ORIGINAL ARTICLE

Promoter hypermethylation of DNA repair genes MLH1 and MSH2 in adenocarcinomas and squamous cell carcinomas of the lung

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Received 27 April 2012; accepted 29 July 2013

KEYWORDS
Adenocarcinoma;
Squamous cell carcinoma;
Lung;
Hypermethylation;
MLH1;
MSH2

Abstract Five years survival of lung cancer is 16%, significantly lower than in prostate (99.9%), breast (88.5%) and colon (64.1%) carcinomas. When diagnosed in the surgical stage it increases to 50% but this group only comprises 14–16% of the cases. DNA methylation has emerged as a potential cancer-specific biomarker. Hypermethylation of CpG islands located in the promoter regions of tumour suppressor genes is now firmly established as an important mechanism for gene inactivation.

This retrospective study included 40 squamous cell carcinomas and 40 adenocarcinomas in various surgical TNM stages to define methylation profile and possible silencing of DNA repair genes – *MLH1* and *MSH2* – using Methylation-Specific PCR and protein expression by immunohistochemistry in tumoural tissue, preneoplastic lesions and respiratory epithelium with normal histological features.

The protein expression of *MLH1* and *MSH2* genes, in the available preneoplastic lesions and in normal cylindrical respiratory epithelium appeared reduced. The frequency of promoter hypermethylation found on these DNA repair genes was elevated, with a higher prevalence of methylation of *MLH1* gene in 72% of squamous cell carcinoma. The differences are not so obvious for *MSH2* promoter hypermethylation. No correlation was found among the status of methylation, the protein expression and the clinicopathological characteristics.

With a larger study, a better characterization of the hypermethylation status of neoplastic and preneoplastic lesions in small biopsies would be achieved, inherent to tumour histology, heterogeneity and preservation, and finally differences in the study population to elucidate other possible mechanisms of altered expression of the *hMLH1* and *hMSH*.
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PALAVRAS CHAVE
Adenocarcinoma;
Carcinoma das células escamosas;
Pulmão;
Hipermetilação;
MLH1;
MSH2

Hipermetilação Promotora de Genes Reparadores de DNA MLH1 e MSH2 em Adenocarcinomas e Carcinomas de Células Escamosas do Pulmão

Resumo A sobrevivência aos cinco anos no cancro do pulmão é de 16%, significativamente inferior que nos carcinomas na próstata (99,9%), mama (88,5%) e cólon (64,1%). Quando diagnosticado na fase cirúrgica aumenta até 50%, mas este grupo é apenas constituído por 14-16% dos casos. A metilação do ADN surgiu como um potencial marcador biológico específico do cancro. A hipermetilação das ilhas CpG localizadas nas regiões promotoras de genes supressores do tumor está agora firmemente estabelecida como um mecanismo importante para a inativação do gene.

Este estudo retrospetivo incluiu 40 carcinomas das células escamosas e 40 adenocarcinomas em vários estádios cirúrgicos TNM para definir o perfil da metilação e o possível silenciamento de genes de reparação do ADN - *MLH1* e *MSH2* - usando metilação PCR específica e expressão da proteína por imuno-histoquímica no tecido tumoral, lesões pré-neoplásicas e epitélio respiratório com características histológicas normais.

A expressão da proteína dos genes *MLH1* e *MSH2*, nas lesões pré-neoplásticas disponíveis e no epitélio respiratório cilíndrico normal, pareceu reduzida. A frequência da hipermetilação promotora encontrada nestes genes reparadores de ADN foi elevada, com uma maior prevalência da metilação do gene *MLH1* em 72% de carcinoma de células escamosas. As diferenças não são tão óbvias para a hipermetilação do promotor *MSH2*. Não foi encontrada correlação entre o estado de metilação, a expressão da proteína e as características clínico-patológicas.

Com um estudo mais amplo, seria alcançada uma melhor caracterização do estado da hipermetilação das lesões neoplásicas e pré-neoplásicas em pequenas biopsias, inerente à histologia, heterogeneidade e preservação do tumor, e, finalmente, às diferenças na população estudada para elucidar outros mecanismos possíveis da expressão alterada do *hMLH1* e *hMSH*.
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Introduction

Epigenetics of human cancer has been overshadowed by human cancer genetics since 1983. Increasingly visible with a growing understanding of specific epigenetic mechanisms and their role in cancer,[1] the modifications refer to a number of molecular mechanisms that regulate gene expression without changing DNA sequence[2]: (1) alteration of methylation status of DNA within CpG islands (the main human epigenetic modification)[3]; (2) covalent modification of histone tails; (3) gene regulation by micro-RNA (miRNA).

While early embryonic cells lack methylation (as it is not transmitted via the germline), methylation is essential for the development and regulation of gene expression and controls expression of oncofetal genes in postnatal life by imprinted genes and tissue-specific gene expression.[2] This perfect equilibrium in normal cells is transformed in cancer cells. DNA methylation is believed to contribute to cancer initiation and progression by gene inactivation. This can have important consequences if the inactivated genes are essential for the control of normal cell growth, differentiation, or apoptosis.[4] The mechanisms that regulate normal and aberrant methylation are neither fully understood nor are the mechanisms of methylation that interfere with transcription.[4]

The mismatch DNA repair (MMR) system is composed of a few well-conserved proteins. The essential components of MMR system, MutS, MutL, MutH and Uvr, were identified in *Escherichia coli*. In addition, all eukaryotic organisms, including humans, have MutS homologs and MutL homologs. The MLH1 and MSH2 genes provide instructions for making a protein that plays an essential role in DNA repair. These proteins fix mistakes that are made when DNA is copied (DNA replication) in preparation for cell division. The MLH1 protein joins with another protein, the PMS2, to form an active protein complex. This protein complex coordinates the activities of other proteins that repair errors during DNA replication. The repairs occur by removing the section of DNA and replacing it by a correct DNA sequence. The MSH2 protein joins with one of the two other proteins, the MSH6 protein or the MSH3 protein, to form an active protein complex. This active protein complex identifies places on the DNA where mistakes have been made during DNA replication.

The prognosis of lung cancer is very limited by the difficulties of diagnosing early stage disease amenable to surgery. Only 10% of cases can benefit from local treatment with long-term survival. Despite much progress in the treatment and detection methods of lung carcinoma, the prognosis remains poor. This situation is mainly the result of metastases which are present in more than two-thirds of patients at the time of diagnosis.[5,6]

The objectives of our study were to characterize the expression of DNA repair proteins MLH1 and MSH2 in tumour tissue, precursor lesions, respiratory epithelia and parenchyma of 80 clinically well-characterized NSCLC patients and also to study the methylation status of two DNA repair genes – *MLH1* and *MSH2*, to correlate between the methylation status of the promoters of *MLH1* and *MSH2*, and their respective protein expression and clinicopathological parameters.

Table 1 MLH1 and MSH2 primer concentrations after optimization.

Gene	Sequence	Tm (°C)	G/C (%)	Conc. (μM)	Amplicon (Bp)
MSH2	M Forward	61	63.2	0.2	
	5′ TCGTGGTCGGACGTCGTTC 3′	64.2	56.5	0.1	114
	M Reverse				
	5′ CAACGTCTCCTTCGACTACACCG 3′				
	U Forward	59.3	41.7	0.4	
	5′ GGTTGTTGTGGTTGGATGTTGTTT 3′	63.9	41.4	0.2	123
	U Reverse				
	5′ CAACTACAACATCTCCTTCAACTACACCA 3′				
MLH1	M Forward	61	45.8	0.1	
	5′ ACGTAGACGTTTTATTAGGGTCGC 3′	61.4	60	0.2	131
	M Reverse				
	5′-CCTCATCGTAACTACCCGCG-3′				
	U Forward	58.2	27.6	0.4	
	5′ TTTTGATGTAGATGTTTTATTAGGGTTG-3′	61.0	45.8	0.2	142
	U Reverse				
	5′ ACCACCTCATCATAACTACCCACA 3′				

Materials and methods

Patients and tissue samples

All biopsy and resection specimens were reviewed by the Pathology Department of the University Hospital of Coimbra, to verify non-small cell histology of the lung cancer samples and to determine the histologic subtype. Histological subtypes consisted of 80 cases of surgically staged pulmonary carcinomas as well as corresponding non-neoplastic and preneoplastic tissue of adenocarcinomas and squamous cell carcinomas of the lung.

Twenty-four females and 16 males with adenocarcinoma (ADC), and 36 males and 4 females with squamous cell carcinoma (SCC) were confirmed according to the latest World Health Organization Classification of Lung Cancer (2004).[7] The average age was 64.9 ± 9.88 years at the time of diagnosis.

The TNM staging was applied according to the 2010 TNM Classification of Malignant Tumours, 7th Edition[8]: thirty-nine patients had Stage I disease, 19 had Stage II and 9 had stage III.

Tissue microarrays building

Representative areas of carcinoma, preneoplastic lesion and normal bronchial epithelium and parenchyma, were carefully selected from the haematoxylin-eosin slide, and marked respectively in each slide and the each formalin-fixed, paraffin-embedded block of tissue. Tissue microarrays of 3 mm were performed in triplicate in tumour and normal respiratory epithelium and in a single core for preneoplastic lesion, included on the same slide.

Analysis of protein expression: immunohistochemistry

IHC satins for hMLH1 and hMLH2 were performed on 3 μm thick sections cut from paraffin-embedded TMAs blocks. Commercially available antibodies against these markers were used as per manufacturer's protocols. Monoclonal antibodies used were (clone ZM001, 1:200, Zymed) for MLH1and (clone FE11, 1:40, Zymed) for MSH2, using the Streptavidin–Biotin Horseradish Peroxidase method. Epitope retrieval using microwave heat or steam pre-treatment was performed as required. Normal endometrium was used as positive control. The normal staining pattern for both MLH1 and MSH2 is nuclear. Lymphocytes and normal bronchial epithelium were used as internal positive control as follow: Normal expression (>75%/+++); Reduced expression: Negative ($\leq$10%/−) and Positive (>10–50%/+ or 50–75%/++).

Following the explained score it was possible to define two definite groups, the positive when present +++/75% nuclear expression and the group with reduced expression. Staining results were examined without knowledge of the status of the molecular analyses.

Methylation-specific polymerase chain reaction assay for the hMLH1 and hMSH2 genes

For DNA extraction, areas from all the slides corresponding to the principal histological patterns were selected. The tumour-rich areas were manually micro-dissected. Five 10 μM sections were cut from the paraffin blocks corresponding to the already selected areas and deparaffined by xylene extraction. The resulting tissue pellets were digested with Proteinase K at 56 °C overnight. The genomic DNA was isolated using QIAamp DNA MiniKit according to the manufacturer' protocol (Qiagen, Valencia, CA). By spectrophotometry (Nanodrop ND1000, Thermo Scientific, USA) the purity and concentration of the samples were verified.

The promoter methylation status of the *MLH1* gene of all tumour samples and of the *MSH2* gene of tumour samples was determined by chemical treatment with sodium bisulfite and subsequent methylation-specific PCR (MSP) analysis as described.[9]

Two sets of primers were specifically designed upstream of the promoter region to discriminate methylated from

unmethylated alleles, using the Methprimer Software[10] (Table 1).

The bisulfite modification was performed using the Epitect Bisulfite Kit (Qiagen Valencia, CA) to convert all unmethylated cytosines to uracils, while leaving methylated cytosines unaffected (Table 2).

Modified DNA was amplified in a total volume of 110 μL containing 1× NH4 buffer, 2 mM of $MgCl_2$, 200 μM of dNTPs, 1 U Taq DNA Polymerase (Bioline), different primer pair concentrations were used after optimization (Table 1), considering that we are working with FFPE tissue, an optimization procedure confers better results. PCR was performed for 40 cycles with annealing temperatures of 61 °C for 30 s and primer extension at 72 °C for 60 s using 50 ng bisulfite-modified DNA. All PCRs were performed with positive controls for both unmethylated and methylated alleles and no DNA control. DNA of healthy human lymphocytes served as positive control for methylated and unmethylated *MLH1* and *MSH2* state. The first was treated with Methyltransferase (CpG Methyltransferase M.SssI) (New England Biolabs), and subsequently submitted to sodium bisulfite transformation (Epitect Bisulfite, Qiagen), the second was only modified with sodium bisulphite. The CpG Methyltransferase, M.SssI, methylates all cytosine residues (C^5) within the double-stranded dinucleotide recognition sequence 5′...CG...3′.

The PCR products were separated on a 4% agarose gel containing ethidium bromide and visualized under UV light, and its size estimated by comparison with a DNA Molecular Weight Marker XIII (50 bp ladder) (Roche).

Statistical analysis

The association between clinicopathological variables (pTNM, gender, age) and frequency of methylation and protein expression silencing among the tumour subtypes was assessed using the Pearson χ^2 and Fischer's exact test. All tests were two-sided and the level of significance was set at $p < 0.05$.

Results

Protein expression of MLH1 and MSH2 in tissue microarrays

We investigated MLH1 and MSH2 expression using immunohistochemistry analysis in tissue microarrays comprising 80 primary NSCLC patients, that were respiratory epithelium, preneoplastic lesion (basal cell hyperplasia, metaplasia, dysplasia) and tumoural patterns. Following the explained score it was possible to define two definite groups, normal expression with >75%/+++ nuclear expression and the group with reduced expression <75%/++. After applying these scores, the internal control of lymphocytes had normal expression (data not shown) but tumour adjacent respiratory epithelium and parenchyma were considered reduced for both MLH1 and MSH2. Twenty-three cases (57%) of ADC for MLH1 expression and 27 cases (67%) for MSH2, had reduced expression; in SCC 29 cases (72%) for MLH1 expression and 25 cases (62%) for MSH2 expression had reduced expression.

Table 2 MLH1 and MSH2 positive immunohistochemistry expression in carcinogenesis progression achieved in tissue microarrays.

	Adenocarcinoma				Squamous cell carcinoma			
	MLH1 expression		MSH2 expression		MLH1 expression		MSH2 expression	
	Normal	Reduced	Normal	Reduced	Normal	Reduced	Normal	Reduced
Respiratory epithelium/parenchyma	7/24 (29.2%)	17/24 (70.8%)	6/24 (25%)	18/24 (75%)	0/35 (0%)	35/35 (100%)	1/36 (2.8%)	35/36 (97.2%)
Basal cell hyperplasia	10/17 (58.8%)	7/17 (41.2%)	7/11 (63.6%)	4/11 (36.4%)	1/6 (16.7%)	5/6 (83.3%)	0/6 (0%)	6/6 (100%)
Epidermoid metaplasia	n.o.	n.o.	n.o.	n.o.	5/17 (29.4%)	12/17 (70.6%)	8/17 (47.1%)	9/17 (52.9%)
Tumour	17/40 (42.5%)	23/40 (57.5%)	13/40 (32.5%)	27/40 (67.5%)	11/40 (27.5%)	29/40 (72.5%)	15/40 (37.5%)	25/40 (62.5%)

n.o., not observed; normal expression 75%/+++; reduced expression positivity below 75%/−.

Table 3 Clinicopathological distribution of gene promoter methylation and loss of expression at tumour type in 80 patients. Patient's age ranging 64.9 ± 9.88 years (63.4 years – adenocarcinoma 66.55 years – squamous cell carcinoma), 28 females and 52 males.

Patient No/age	Gender (M/F)	Tumour Type	TNM Stage	MLH1 Loss Expression	*MLH1* Methylation	MSH2 Loss Expression	*MSH2* Methylation
1/73	M	ADC	pT1N0Mx IA				
2/71	F	ADC	T1NxMx				
3/69	M	ADC	pT2N0Mx IB				
4/75	M	ADC	pT1N2Mx; IIIA				
5/53	F	ADC	pT1N0 Mx IA				
6/76	M	ADC	pT2N0Mx IB				
7/62	M	ADC	pT1N1Mx IIA				
8/74	F	ADC	n.a.				
9/50	F	ADC	pT1N1Mx IIA				
10/68	F	ADC	pT1N0Mx IA				
11/57	F	ADC	pT2N0Mx IB				
12/51	M	ADC	pT1N1Mx IIA				
13/55	F	ADC	n.a.				
14/75	F	ADC	pT2N0Mx IB				
15/85	M	ADC	pT1NxMx				
16/63	F	ADC	pT1N2Mx IIIA				
17/67	F	ADC	n.a.				
18/48	M	ADC	n.a.				
19/71	M	ADC	pT2N1Mx IIB				
20/63	F	ADC	pT1N2Mx IIIA				
21/49	M	ADC	pT3N0Mx IIB				
22/80	F	ADC	pT1NxMx				
23/68	F	ADC	pT2N0Mx IB				

Table 3 (*continued*)

24/48	M	ADC	pT2N0Mx IB				
25/56	M	ADC	pT1N0Mx IA				
26/76	M	ADC	pT1N0Mx IA				
27/64	M	ADC	pT2N0Mx IB				
28/56	F	ADC	pT1N0Mx IA				
29/67	F	ADC	pT2N0Mx IB				
30/71	F	ADC	pT2N0Mx IB				
31/50	F	ADC	pT1N0Mx IA				
32/80	F	ADC	n.a.				
33/56	F	ADC	pT1N0Mx IA				
34/54	F	ADC	pT1N0Mx IA		n.d.		n.d.
35/67	M	ADC	pT2N1Mx IIB		n.d.		n.d.
36/62	F	ADC	pT1N1Mx IIA		n.d.		n.d.
37/44	F	ADC	pT2N2Mx IIIA		n.d.		n.d.
38/52	F	ADC	pT4N1Mx IIIB		n.d.		n.d.
39/61	M	ADC	pT2N0Mx IB		n.d.		n.d.
40/68	F	ADC	pT1NxMx		n.d.		n.d.
41/66	M	SCC	pT1N0Mx IA				
42/69	M	SCC	pT1N0Mx IA				
43/68	M	SCC	pT2N0Mx IB				
44/70	M	SCC	pT2N0Mx IB				
45/73	M	SCC	pT2N0Mx IB				
46/65	M	SCC	pT3N0Mx IIB				
47/53	M	SCC	pT2N0Mx IB				
48/61	M	SCC	pT1N0Mx IA				
49/48	M	SCC	pT1N1Mx IIA				
50/65	M	SCC	pT2N0Mx IB				
51/53	M	SCC	pT1NxMx				
52/84	M	SCC	pT2N0Mx IB				

Table 3 (*continued*)

53/73	M	SCC	pT2N0Mx IB				
54/61	M	SCC	pT2N2Mx IIIA				
55/77	M	SCC	pT2N0Mx IB				
56/59	M	SCC	pT2N1Mx IIB				
57/52	M	SCC	pT2N0Mx IB				
58/72	M	SCC	n.a.				
59/55	M	SCC	pT2N1Mx IIB				
60/80	M	SCC	pT2N0Mx IB				
61/79	M	SCC	pT3N0Mx IIB				
62/74	M	SCC	n.a.				
63/50	M	SCC	pT3N1Mx IIIA				
64/80	M	SCC	pT1N0Mx IA				
65/73	M	SCC	pT2N1Mx IIB				
66/74	M	SCC	pT1N0Mx IA				
67/72	F	SCC	pT2N0Mx IB				
68/77	M	SCC	pT4N0Mx IIIB				
69/65	M	SCC	pT2N1Mx IIB				
70/65	M	SCC	pT3N0Mx IIB				
71/72	M	SCC	pT2N1Mx IIB				
72/68	M	SCC	pT2N1Mx IIB				
73/60	M	SCC	pT3N2Mx IIIA				
74/67	M	SCC	pT1N0Mx IA				
75/69	M	SCC	pT1N0Mx IA				
76/53	F	SCC	n.a.				
77/58	M	SCC	pT2N1Mx IIB				
78/71	M	SCC	pT3N0Mx IIB				
79/61	F	SCC	pT2N0Mx IB				
80/70	F	SCC	pT2N0Mx IB				

White grey denotes unmethylated and normal protein expression respectively, dark grey methylated promoter genes. M, male; F, female; ADC, adenocarcinoma; SCC, squamous cell carcinoma; T, tumour size; N, local metastasis; M, distant metastasis; n.a., not available; n.d., not determined.

Co-reduction of both MLH1 and MSH2 was found in 38 cases (19 ADC and 19 SCCs), according with Table 5, probably due to different altered epigenetic or/and genetic mechanisms involved in down-regulation of gene expression. Tumour cells that exhibited an absence of nuclear staining in the presence of non-neoplastic cells and infiltrating lymphocytes with nuclear staining were considered to have an abnormal pattern.

In normal respiratory epithelium reduced expression was found in 70% MLH1 and 75% MSH2 in ADC, and 100% MLH1

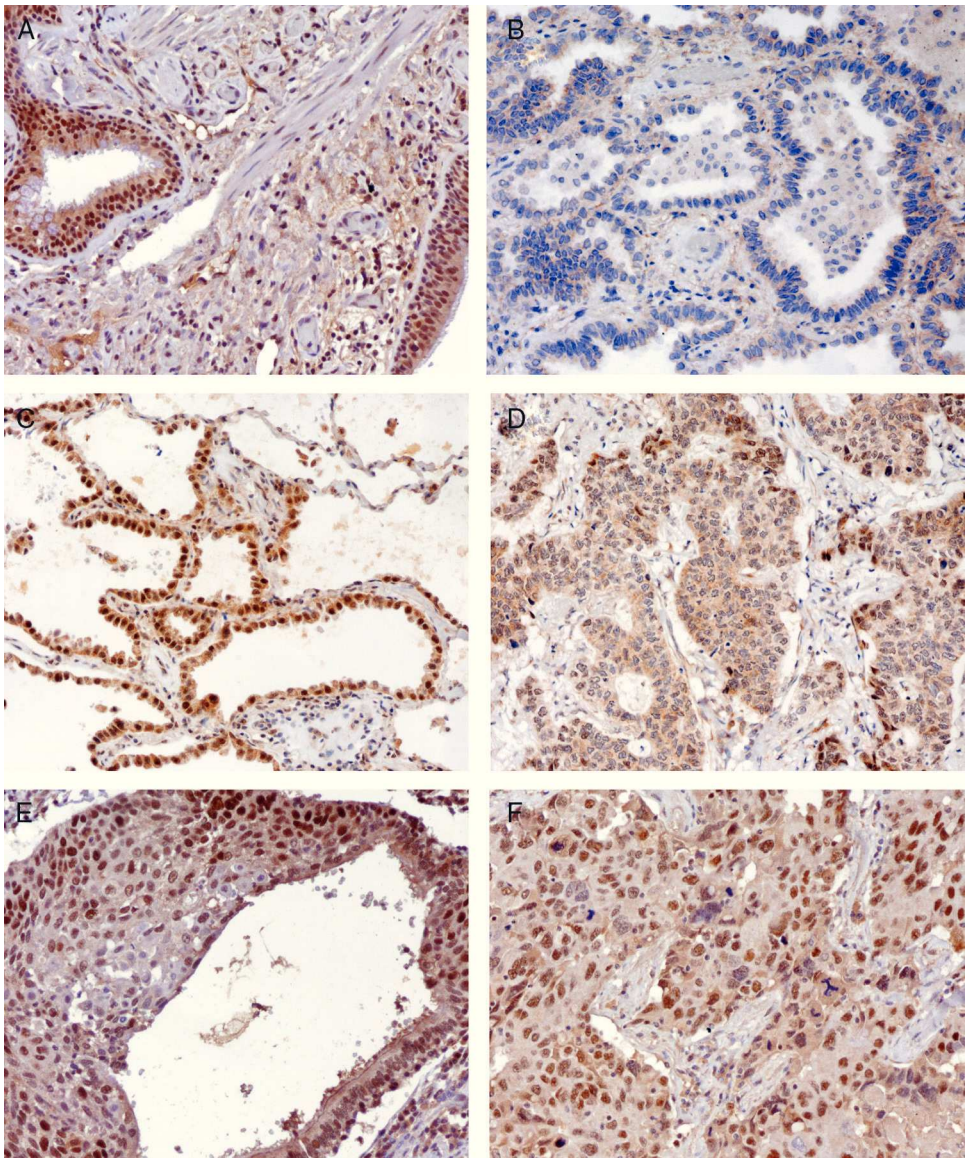

Figure 1 MLH1 IHC expression in one mixed type adenocarcinoma, (A) basel cell hyperplasia, (B) acinar pattern with reduced expression (−), (C) bronchioloalveolar pattern with normal expression (+++) and (D) solid pattern with reduced expression (++); MSH2 IHC expression in epidermoid carcinoma in situ and (E) corresponding epidermoid carcinoma (F) 200×.

and 97% in MSH2 in SCC, to study this we would need cancer-free controls to explain the possible time required to acquire the additional genetic and epigenetic changes that promote tumour progression.

Basal cell hyperplasia in cases of adenocarcinoma presented normal expression of MLH1 and MSH2. Squamous cell carcinomas revealed reduced expression of MLH1 and MSH2 in 72% and 62.5% respectively. Fig. 1 represents the IHC expression heterogeneity.

MLH1 and *MSH2* methylation status in tumour samples

We examined the promoter hypermethylation status of *MLH1* and *MSH2* genes in 40 squaumous cell carcinoma and 33 adenocarcinoma using MSP assay in manually microdissected tissue. 40 DNA samples of squamous cell carcinoma and 33 samples of adenocarcinoma were analyzed (Table 3).

We observed two patterns of hypermethylation; the partial pattern was the most prevalent (58.33%) for the *MLH1* gene (M-U) while for the *MSH2* gene the hypermethylation fully pattern (M-M) was the one most verified (76.9%).

MLH1 methylation pattern seems to vary substantially by histological type (Table 3). The prevalence of *MLH1* promoter hypermethylation was higher in squamous cell carcinoma (47.5%), with a correlation ($p = 0.003$) between the histological subtype and the methylation status of the promoter of *MLH1* gene. The difference is not so obvious in *MSH2* promoter hypermethylation; however adenocarcinoma has the highest number of cases (42.4%). No correlation was found between histological subtype and methylation status of *MSH2* ($p = 0.2699$).

Table 4 Promoter hypermethylation frequency in NSCLC in tumour tissues, by histological types.

	ADC ($N = 33$)	SCC ($N = 40$)
MLH1	5 (15.2%)	19 (47.5%)
MSH2	14 (42.4%)	12 (30%)

According to the relationship between the frequency of gender and histological subtype, women with adenocarcinoma as well as men with squamous cell carcinoma have a higher percentage of hypermethylation of *MLH1* and *MSH2* promoters (Table 4).

Relationship between the methylation status and IHC protein expression

According to the distribution of gene promoter methylation and loss of expression at tumour type in 80 patients, no statistical correlation was observed between the promoter methylation and expression levels in tumour samples, $p = 0.09$ for MLH1 and $p = 0.894$ for MSH2 (Tables 5–7).

Moreover, no correlation was found between *MLH1* and *MSH2* methylation levels and any of the clinicopathological characteristics considered (age, gender, TNM stage and tumour histological type) (Table 7).

We observed two patterns of hypermethylation; the partial pattern was the most prevalent (58.33%) for the *MLH1* gene (M-U) while for the *MSH2* gene the hypermethylation fully pattern (M-M) was the one most verified (76.9%).

Discussion

Most clinical studies, have recorded moderate to high elevation of MSH2 expression in sporadic tumours like colon, prostate or bladder while there is substantial reduction to near loss of functional MLH1 or MSH2 protein expression in ovarian, lung and stomach tumours relative to their matched normal tissues.[11–13] Reduced expression of MLH1 and MSH2 was observed in respiratory epithelia, metaplasia and dysplasia. We know that cancer is a disease involving multiple pathways and genetic lesions, which are necessary for a tumour to become fully established. The story is the same for epigenetic lesions. It is difficult to explain the differential rates of progression of premalignant lesions and differences in behaviour of morphologically similar lesions. Heterogeneity for microsatellite instability (MSI) and promoter methylation in driving these phenomena forward may explain this; however, no previous analysis has examined this in detail.

Alterations in expression of MMR proteins MLH1 and MSH2 have been reported in a variable proportion of NSCLC but very few studies have investigated the role of reduced protein expression in precursor lesions of NSCLC or have investigated their potential prognostic significance in invasive carcinomas. More recent studies have identified changes in methylation in the oral epithelium of smokers and linked these changes to bronchial epithelium.[14,15]

We found differences concerning the pattern of methylation of *MLH1* gene depending on the histological typing of NSCLC ($p = 0.003$) and within the subtype. Fifteen percent of the ADC cases showed hypermethylation of *MLH1* compared with 47.5% cases of methylated SCC. Although we found no significant differences in the methylation pattern of *MLH1* and *MSH2* genes in SCC, ADC has a higher frequency of *MLH1* methylated rather than *MSH2*.

Table 7 Correlation of *MLH1* and *MSH2* methylation levels and respective protein expression in relation to the clinicopathological variables of NSCLC tumours (age, gender and TNM stage).

	Age	Gender	TNM stage
NSCLC			
MLH1 loss expression	0.319	0.555	0.988
MLH1 methylation	0.077	0.920	0.133
MSH2 loss expression	0.669	0.555	0.189
MSH2 methylation	0.737	0.139	0.562

Table 5 Frequency of promoter hypermethylation in NSCLC tumour tissue, by histological type and gender.

	ADC		SCC	
	Female ($N = 19$)	Male ($N = 14$)	Female ($N = 4$)	Male ($N = 36$)
MLH1	4 (80%)	1 (20%)	2 (10.5%)	17 (89.5%)
MSH2	8 (57.1%)	6 (42.9%)	3 (25%)	9 (75%)

Table 6 Correlation between MLH1 and MSH2 promoter hypermethylation and its possible interference in respective immunohistochemistry expression.

IHC expression	*MLH1* promoter hypermethylation		*MSH2* promoter hypermethylation	
	M	U	M	U
Normal expression	5	20	9	16
Reduced expression	19	29	17	31
P	0.09		0.894	

In patients with ADC we also found basal cell hyperplasia that presents MLH1 and MSH2. Although this can be explained by the fact that the pathogenesis of adenocarcinoma is not related with the bronchus and therefore with basal cells, but with Clara Cells and type-II pneumonocytes, so basal cell hyperplasia and also metaplasia are not required to have reduced protein expression.[16]

Females with adenocarcinoma were significantly more likely to have methylated *MLH1* and *MSH2* compared to males. Although, these differences were not observed in squamous cell carcinoma, males were more likely to have hypermethylation of *MLH1* and *MSH2*. These substantial differences have not been consistently noted in the literature,[15] at least on the genes under study, perhaps because few studies have been stratified by histological type when assessing hypermethylation differences by gender.[15] Since females are more likely to have adenocarcinomas compared to males, differences in hypermethylation frequency due to gender may have been obscured by differences due to histological type.[16]

Hawes et al. mentioned in their recent work that substantial differences in hypermethylation by gender may suggest the possibility of differential pathways and/or risk factors for NSCLC between genders, with respect to risk factors, especially with regards to the effect of cigarette smoking as well as survival and effectiveness of treatment.[17,18] Hormonal factors may account for these differences, although the mechanism that influences the methylation status in NSCLC remains unclear.[18]

As in our study, Xinarianos et al.[19] and Cooper et al.,[20] did not find any correlation between reduced MLH1 and MSH2 expression and age, gender, tumour differentiation or TNM stage.

Most of the studies used qualitative methylation-specific PCR in order to detect DNA methylation, a method that can sometimes lead to false positive results and does not distinguish between low and high level methylation. Furthermore, results in these studies have been inconsistent due to varying methylation detection protocols, PCR primers, and study populations, and none have comprehensively studied more than 10 genes.[16,17]

The variability in results that we observed is probably due to a number of technical factors, such as assay-specific differences including target sites of CpG island loci, primers and the conditions of sodium bisulfite modification. Promoter hypermethylation is one of the mechanisms responsible for the genes silencing,[4] although in this work no correlation was found between *MLH1* and *MSH2* promoter methylation and IHC expression in tumour samples (Tables 6 and 7).

To better understand the role of MMR system in the tumourigenesis of carcinomas of the lung, we conclude that there is a real need for a more robust study covering the differences inherent to tumour histology, heterogeneity and preservation, and finally differences in the study population (age, gender and number of subtyped cases beyond NSCLC), to elucidate other possible mechanisms of altered expression of the *hMLH1* and *hMSH2*, including loss of heterozygosity, chromosomal instability and imbalance mutations in genes of the MMR system,[8] and alterations in mRNA transcription.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that they have followed the protocols of their work centre on the publication of patient data and that all the patients included in the study received sufficient information and gave their written informed consent to participate in the study.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Funding

Funded by a grant from CIMAGO, Faculty of Medicine, University of Coimbra, Portugal.

Conflicts of interest

The authors have no conflicts of interest to declare.

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