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Corresponding Author: Dr Maija Kohonen-Corish,

Corresponding Author's Institution: Garvan Institute of Medical Research

First Author: Pirooz Poursoltan

Order of Authors: Pirooz Poursoltan; Laurent Pangon; Nicola Currey; Christa van Kralingen; Annabelle Mahar; Christina I Selinger; Wendy A Cooper; Catherine W Kennedy; Brian C McCaughan; Ronald Trent; Maija Kohonen-Corish

Abstract: 'Mutated in colorectal cancer' (MCC) is emerging as a multifunctional protein that affects several cellular processes and pathways. Although the MCC gene is rarely mutated in colorectal cancer, it is frequently silenced through promoter methylation. Previous studies have reported loss of heterozygosity (LOH) of the closely linked MCC and APC loci in both colorectal and lung cancers. APC promoter methylation is a marker of poor survival in non-small cell lung cancer (NSCLC). However, MCC methylation has not been previously studied in lung cancer. Therefore, we wanted to determine if MCC is silenced through promoter methylation in lung cancer and whether this methylation is associated with LOH of the MCC locus and/or methylation of the APC gene. Three polymorphic markers for the APC/MCC locus were analysed for LOH in 64 NSCLC specimens and matching normal tissues. Promoter methylation of both genes was determined using methylation specific PCR in primary tumours. LOH of any of the three markers was found in 55% of the specimens. LOH within the MCC locus was less common in adenocarcinoma (ADC) (29%) than in squamous cell carcinoma (SCC) (76%; P=0.004) or large cell carcinoma (LCC) (75%; P=0.014). However, this LOH was not accompanied by MCC promoter methylation, which was found in only two cancers (3%). In contrast, 39% of the specimens showed APC methylation, which was more common in ADC (58%) than in SCC (13%). Western blotting revealed that MCC was expressed in a subset of lung tissue specimens but there was marked variation between patients rather than between cancer and matching non-cancer tissue specimens. In conclusion, we have shown that promoter methylation of the APC gene does not extend to the neighbouring MCC gene in lung cancer, but LOH is found at both loci. The variable levels of MCC expression were not associated with promoter methylation and may be regulated through other cellular mechanisms.

Suggested Reviewers: Kwun Fong
The Prince Charles Hospital UQ Thoracic Research Centre, Brisbane, Australia
fongk@health.qld.gov.au
Expert on epigenetic biomarkers in lung cancer.

Peter W Laird
University of Southern California/Norris Comprehensive Cancer Center, Los Angeles, California, USA.
plaird@usc.edu
Expert on epigenetic markers of cancer.

Dr Maija Kohonen-Corish
Cancer Institute NSW Fellow
Group Leader, Cancer Genetics & Biology
Cancer Research Program
Email m.corish@garvan.org.au
Tel +61-2-9295 8336
Fax +61-2-9295 8321

GARVAN INSTITUTE OF MEDICAL RESEARCH
384 VICTORIA STREET
DARLINGHURST SYDNEY
NSW 2010 AUSTRALIA

Lung Cancer Editorial Office
Elsevier House, Brookvale Plaza
East Park, Shannon, Co. Clare
Ireland
Tel: +353 61 709640
Fax: +353 61 709107

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Loss of heterozygosity of the 'Mutated in Colorectal Cancer' gene is not associated with promoter methylation in non-small cell lung cancer

Dear Professor Stahel,

Please find attached our manuscript, which we wish to submit for consideration by **Lung Cancer**. In this paper we describe our original findings on the genetic and epigenetic alterations of the 'Mutated in Colorectal Cancer' (MCC) gene in lung cancer.

This manuscript, including related data, figures and tables has not been published previously. The manuscript is not currently under consideration elsewhere.

The MCC gene was initially identified in 1991 due to its close linkage with the APC gene that was shown to be the susceptibility locus for familial adenomatous polyposis. Early reports described loss of heterozygosity (LOH) of both the APC and MCC genes in lung cancer. Later it was found that the APC gene is silenced through promoter methylation in lung cancer, but the MCC gene was largely overlooked for many years.

We described for the first time in 2007 that the MCC gene is commonly silenced in colorectal cancer through promoter methylation, thus rediscovering the importance of this gene as a candidate tumour suppressor gene. MCC is now emerging as a multifunctional protein that affects several cellular processes and pathways and its significance as a tumour suppressor has been confirmed in a mouse model of colorectal cancer. Therefore, we are in an ideal position to show that MCC methylation does not play a role in lung carcinogenesis, while also confirming the previous LOH findings.

As there are still relatively few publications on MCC, we believe that this would become a highly cited paper.

Word count 2000.

Yours sincerely
Maija Kohonen-Corish

Loss of heterozygosity of the *Mutated in Colorectal Cancer* gene is not associated with promoter methylation in non-small cell lung cancer

Pirooz Poursoltan[1,2], Laurent Pangon[3], Nicola Currey[3], Christa van Kralingen[3], Christina I Selinger[3,4], Annabelle Mahar[4], Wendy A Cooper[4,5], Catherine W Kennedy[6], Brian C McCaughan[7], Ronald Trent[1,2], Maija R J Kohonen-Corish[3,5,8]

[1] Department of Molecular and Clinical Genetics, Royal Prince Alfred Hospital, Missenden Road, Camperdown, Sydney, NSW 2050, Australia

[2] Sydney Medical School, University of Sydney, NSW 2006, Australia

[3] Cancer Research Program, Garvan Institute of Medical Research, 384 Victoria Street, Darlinghurst, Sydney, NSW 2010 Australia

[4] Tissue Pathology and Diagnostic Oncology, Royal Prince Alfred Hospital, Missenden Road, Camperdown, Sydney, NSW 2050, Australia

[5] School of Medicine, University of Western Sydney, NSW 2560, Australia

[6] Strathfield Private Hospital, Everton Road, Strathfield NSW 2135, Australia

[7] Department of Cardiothoracic Surgery, Royal Prince Alfred Hospital, Missenden Road, Camperdown, NSW 2050, Australia

[8] St Vincent's Clinical School, Faculty of Medicine, University of New South Wales, Sydney, NSW 2052, Australia

Running title: LOH and promoter methylation of the MCC and APC genes in lung cancer

Correspondence
Maija Kohonen-Corish
Cancer Research Program, Garvan Institute of Medical Research
384 Victoria St, Darlinghurst, NSW, 2010
Ph: +61 292958336 Fax: +61 292958321
m.corish@garvan.org.au

Abstract

'*Mutated in colorectal cancer*' (*MCC*) is emerging as a multifunctional protein that affects several cellular processes and pathways. Although the *MCC* gene is rarely mutated in colorectal cancer, it is frequently silenced through promoter methylation. Previous studies have reported loss of heterozygosity (LOH) of the closely linked *MCC* and *APC* loci in both colorectal and lung cancers. *APC* promoter methylation is a marker of poor survival in non-small cell lung cancer (NSCLC). However, *MCC* methylation has not been previously studied in lung cancer. Therefore, we wanted to determine if *MCC* is silenced through promoter methylation in lung cancer and whether this methylation is associated with LOH of the *MCC* locus and/or methylation of the *APC* gene. Three polymorphic markers for the *APC/MCC* locus were analysed for LOH in 64 NSCLC specimens and matching normal tissues. Promoter methylation of both genes was determined using methylation specific PCR in primary tumours. LOH of any of the three markers was found in 55% of the specimens. LOH within the *MCC* locus was less common in adenocarcinoma (ADC) (29%) than in squamous cell carcinoma (SCC) (76%; P=0.004) or large cell carcinoma (LCC) (75%; P=0.014). However, this LOH was not accompanied by *MCC* promoter methylation, which was found in only two cancers (3%). In contrast, 39% of the specimens showed *APC* methylation, which was more common in ADC (58%) than in SCC (13%). Western blotting revealed that MCC was expressed in a subset of lung tissue specimens but there was marked variation between patients rather than between cancer and matching non-cancer tissue specimens. In conclusion, we have shown that promoter methylation of the *APC* gene does not extend to the neighbouring *MCC* gene in lung cancer, but LOH is found at both loci. The variable levels of MCC expression were not associated with promoter methylation and may be regulated through other cellular mechanisms.

Key words: non-small cell lung cancer; *APC*; *MCC*; promoter methylation; LOH; loss of heterozygosity

Introduction

Allelic loss of specific chromosomal regions is a common molecular change in non-small cell lung cancers (NSCLC) [1]. Early studies of loss of heterozygosity (LOH) in NSCLC and other cancers led to the identification of several important tumour suppressor genes that reside in these regions. In certain tumour suppressor loci, LOH is accompanied by promoter methylation, which leads to complete silencing of the gene. Promoter methylation is particularly common in NSCLCs, which display concordant methylation of multiple genes, and can be detected with PCR-based techniques in sputum and blood [2]. This has led to efforts to develop new methylation biomarker tests for the early detection of lung cancer [3]. Some chromosomal regions are subject to long-range epigenetic regulation, where adjacent genes are co-ordinately suppressed during carcinogenesis, such as 3p22 region in colon cancer [4], bladder cancer [5] and possibly lung cancer [6].

One of the genomic regions of interest in lung cancer is 5q21-22, which houses the *Adenomatous Polyposis Coli* (*APC*) and the *Mutated in Colorectal Cancer* (*MCC*) genes. *APC* was identified in 1991 as the susceptibility gene for familial adenomatous polyposis (FAP) [7] and is also commonly mutated in sporadic colorectal cancer (CRC) [8]. In lung cancer the *APC* gene is methylated in at least 53% of NSCLC [9, 10], but the significance of the *APC* defect in lung cancer is still poorly understood.

The *MCC* gene was discovered during the search for the FAP locus because of its close linkage to the *APC* gene [11]. Although LOH of the *MCC* gene was found to be frequent in both colorectal and lung cancers [12], it was subsequently discovered that the remaining *MCC* allele was only rarely mutated in CRC [13, 14]. Therefore, a possible tumour suppressor role of *MCC* in carcinogenesis was not seriously pursued until we demonstrated that *MCC* promoter methylation is common in CRC, involving up to 50% of primary cancers [15]. This methylation involves the main short isoform of *MCC* and is

particularly common (80%) in serrated polyps [15], which are thought to be the precursors of CRC in the serrated neoplasia pathway [16]. A subsequent study reported that MCC inhibits beta-catenin transcriptional activity in CRC cells, thus mimicking the important tumour suppressor function of APC, although through a different mechanism [17]. These findings have rekindled interest in the role of MCC in carcinogenesis but it has not been previously determined if the *MCC* promoter is also methylated in lung cancer.

The 5q21-22 region affected by LOH in lung cancers includes both *APC* and *MCC* [12, 18]. LOH of the *APC/MCC* locus is associated with poor survival in patients with NSCLC or the subset of patients with squamous cell carcinoma [18]. There is also previous evidence from mouse models of lung cancer that MCC expression is decreased or lost during lung carcinogenesis [19, 20]. Therefore, we wanted to determine if *MCC* is silenced through promoter methylation in lung cancers and whether this methylation is associated with LOH in the *MCC* locus and/or methylation of the *APC* gene.

Materials and Methods

Lung cancer patients

Lung cancer and matching normal tissue specimens were obtained from surgical resections [6, 21]. Ethical approval for the study was given by the Ethics Review Committee at the Royal Prince Alfred Hospital (X06-0167, X10-0278 and HREC/10/RPAH/491). *APC/MCC* LOH and methylation was analysed in 64 patients including 43 stage 1-2, and 21 stage 3-4 tumours. *APC* methylation was also determined in a larger cohort of stage 1 and 2 NSCLC (239 patients) previously analysed for a number of other epigenetic markers [6, 21]. MCC protein expression was analysed in prospectively collected tissue specimens from 14 patients.

Protein expression

Cell lines H460, H292, H1299, H358, A549 and H520 were obtained from ATCC (Manassas, VA, USA). Patient specimens of cancer and matching normal tissue were biopsied from surgical resections by an experienced histopathologist (AM, WC) and frozen in -80°C. Cell lysates were analysed with western blotting using the following antibodies: anti-MCC monoclonal (610740, BD Transduction Laboratories™, Franklin Lakes, NJ USA), and GAPDH (4300, Ambion, Austin, TX, USA).

APC and MCC methylation

Genomic DNA, extracted from formalin fixed paraffin embedded specimens, was treated with bisulfite as previously described [6]. *APC* methylation and the internal reference gene *MYOD1* were analysed following the MethyLight protocol [15, 22, 23]. Methylation of the short MCC isoform promoter was analysed using a manual methylation specific PCR protocol [15].

Loss of heterozygosity

Two microsatellite markers D5S346 and D5S656 and an insertion-deletion polymorphism in intron 10 of the *MCC* gene were used to analyse LOH. Genomic DNA was extracted from formalin fixed paraffin embedded specimens [6]. PCR products were analysed with capillary electrophoresis (ABI PRISM 310 Genetic Analyser) and the size ratio of the two alleles in cancer tissue was compared to matched normal lung tissue. A 40% reduction in the ratio of allelic sizes was considered as the threshold for LOH of the marker. D5S656 is located in the 5' flanking region of the *MCC* short isoform and has eight alleles, 185-203 bp. D5S346 is located in the 3' flanking region of *APC* between *APC* and *MCC.* This has been used as a marker for the *APC* gene in previous studies due to its proximity to this

gene [24]. This marker contains 13 alleles, 96-122 bp. The insertion/deletion polymorphism in *MCC* intron 10 (INT10) has two alleles, 79 and 93 bp [25]. The order of the markers in the chromosome is D5S346-INT10-D5S656.

Statistical and survival analysis

Correlation between the different parameters was determined using the chi square test. Survival analysis was performed with the Kaplan-Meier log-rank test in the StatView package as previously described [6]. P=0.05 was regarded as statistically significant.

Results

Loss of heterozygosity of the MCC locus is more frequent in Squamous Cell Carcinoma and Large Cell Carcinoma than in Adenocarcinoma.

Of the three markers, INT10 and D5S656 are within the *MCC* genomic locus whereas the D5S346 marker is located between the *APC* and *MCC* genes. The overall frequency of LOH in NSCLC was 48-49% for the two *MCC* markers and 41% for D5S346. LOH of either of the two markers within the *MCC* locus was less common in adenocarcinomas (ADC) (29%) than in squamous cell carcinomas (SCC) (76%; P=0.004) or in large cell carcinomas (LCC) (75%; P=0.014) (Tables 1-3). Also, LOH of the D5S346 marker was less common in ADC (24%) than in SCC (58%; P=0.032). LOH of at least one marker was detected in 47% of stage 1-2 and in 71% of stage 3-4 lung cancer specimens, showing a trend towards an increase of LOH during cancer progression (P=0.065).

MCC promoter methylation is rare in NSCLC

MCC and *APC* methylation could be assessed in 57 out of the 64 specimens analysed for LOH (Tables 1-3). Methylation of *MCC* was found in only two cancers (3%), including

one LCC and one ADC. In contrast 22 specimens had *APC* methylation (39%) and one of them also showed *MCC* methylation (LCC). No methylation of either gene was detected in the matching normal bronchial tissue specimens. *APC* methylation was more common in ADC (58%) than in SCC (13%; P=0.005) and there was no association with LOH of the D5S346 locus. Only 6 out of 21 cancers with LOH of this marker showed *APC* methylation.

We next analysed a larger cohort of stage 1 and 2 patients to assess whether *APC* methylation was associated with poorer survival as previously reported [10]. *APC* methylation was found in 90 out of 239 cancers (40%) and was associated with lower AJCC6 stage (P=0.046) and ADC (P=0.005), but negatively associated with SCC (P=0.0005). There was no association between *APC* methylation and survival in either the whole NSCLC cohort or in the patient subgroups with ADC, SCC or LCC.

MCC protein expression is variable in lung cancer cell lines and primary lung cancers

Although our results indicated that *MCC* methylation is a rare event in NSCLC, it is possible that *MCC* could be downregulated through mutational or other cellular mechanisms. Therefore, we next examined by Western blot if there was any evidence of loss of MCC protein expression in a series of primary NSCLC tumours and matching macroscopically normal tissue specimens (Fig 1a). No loss of MCC expression was seen in the tumours compared to the matching non-tumour tissue specimens. However, there was marked variation between patients in the level of overall MCC expression. We then analysed a series of lung cancer cell lines (Fig 1b). H520 (SCC) cells had no MCC whereas H460 (LCC) cells showed strong MCC expression. Other cell lines H292, H1299, H358 and A549 showed varying degrees of MCC expression. The mucoepidermoid carcinoma

H292 and metastatic NSCLC H1299 cells showed both the long and the short protein isoforms of MCC. None of the lung cancer cell lines had any evidence of methylation of the *MCC* gene promoter that was seen in colorectal cancer specimens [15]. Therefore it appears MCC expression is highly variable in lung cancer and the adjacent non-tumour tissue but this is not associated with cancer-specific promoter methylation.

Discussion

MCC is emerging as a multifunctional protein that affects several cellular processes and pathways. In addition to regulating cell proliferation in many cancer cell lines [17, 26], it has been suggested that MCC is involved in differentiation [27], epithelial cell migration [28], DNA damage response [29] and inhibition of NFκB activation [30] or Wnt signalling [17]. Importantly, the tumour suppressor role of *MCC* has been confirmed in a mouse model of colon cancer [31]. It is well established that *MCC* is epigenetically silenced in sporadic CRC [15, 17], but it has not been previously investigated if the *MCC* gene is also methylated in lung cancer leading to loss of protein expression.

This study has shown that LOH of the *MCC* locus is common in NSCLC but is not accompanied by *MCC* promoter methylation, which is rare in NSCLC. This suggests that methylation silencing of *MCC* transcription is not a significant feature of carcinogenesis in NSCLC. However, we also found that the relatively frequent *APC* methylation is not associated with LOH within the *MCC/APC* locus. As we did not study more markers within the *APC* locus, the significance of this finding is unclear. Therefore, it remains a possibility that LOH occurs independently in the *APC* and *MCC* loci as has been previously suggested in esophageal cancer [32].

Our findings confirm and extend previous studies of the *APC/MCC* locus in NSCLC. There were differences between SCC and ADC in the frequency of LOH and

APC methylation. LOH was more common in SCC than in ADC, whereas the reverse applied for *APC* methylation. Fong et al [18] also found that LOH of the *APC/MCC* locus was more common in SCC than in ADC, and Saito et al [33] recently reported that the level of *APC* methylation was lowest in SCC. Interestingly, we could not confirm that *APC* methylation is associated with poorer survival in this cohort but this could be related to the size of the cohort or the specific mix of different NSCLC subtypes. These patients have been previously analysed for other epigenetic markers, such as *DLEC1* methylation and SATB1 loss of protein expression, which were found to be independent markers of poor survival [6, 21].

Although no *MCC* methylation was observed in primary lung cancers or cell lines, Western blot analysis revealed that MCC protein expression was highly variable even in non-tumour tissue from lung cancer patients. Further analysis of healthy individuals is required to determine the normal level of MCC expression in different lung tissues. In conclusion, we have shown that the promoter methylation observed in the *APC* gene does not extend to the neighbouring *MCC* gene, but LOH is found at both loci. The level of MCC expression in lung cancer appears to be regulated through other cellular mechanisms.

Acknowledgements

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Conflict of interest statement

None declared.

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Figure 1. Western blot analysis of primary NSCLC and matching non-tumour tissue (A) and NSCLC cell lines (B). No expression of MCC was detected in H520 cells, low expression in A549 and higher expression in H460, H292 and H1299 cells. Specimens 1, 4, 5, 13 and 15 are from SCC; 3, 6, 8, 10, 12, 14 and 16 are from ADC; 11 from LCC and 7 from a combined small cell and large cell neuroendocrine carcinoma. T= tumour tissue, N= adjacent non-cancer tissue. GAPDH and beta-actin are loading controls.

Table 1. Loss of heterozygosity (LOH) and promoter methylation of the MCC and APC genes in adenocarcinomas.

Patient ID	D5S346	INT10	D5S656	MCC met	APC met
84	LOH	LOH	LOH	U	M
237	LOH	LOH	No LOH	U	U
228	LOH	LOH	NR	NR	NR
225	LOH	NI	LOH	NR	NR
219	LOH	NI	NR	NR	NR
252	LOH	NR	No LOH	U	U
136	LOH	NR	NR	M	U
166	No LOH	LOH	NI	U	M
41	No LOH	LOH	No LOH	U	U
75	No LOH	NI	LOH	U	M
21	No LOH	NI	NI	U	U
170	No LOH	NI	NI	U	U
162	No LOH	NI	NI	U	M
5	No LOH	NI	No LOH	U	M
31	No LOH	NI	No LOH	U	U
154	No LOH	NI	No LOH	U	U
254	No LOH	NI	No LOH	U	M
6	No LOH	NI	No LOH	U	U
135	No LOH	NI	No LOH	U	M
217	No LOH	No LOH	NI	U	M
9	No LOH	No LOH	No LOH	U	M
24	No LOH	No LOH	No LOH	U	M
93	No LOH	No LOH	No LOH	U	U
140	No LOH	No LOH	No LOH	U	M
169	No LOH	No LOH	No LOH	U	M
230	No LOH	No LOH	No LOH	U	M
15	No LOH	No LOH	No LOH	U	M
165	No LOH	No LOH	NR	U	M
245	No LOH	No LOH	NR	U	U

NI=not informative; M=methylation detected; U=no methylation detected; NR=no result

Table 2. Loss of heterozygosity (LOH) and promoter methylation of the MCC and APC genes in squamous cell carcinomas.

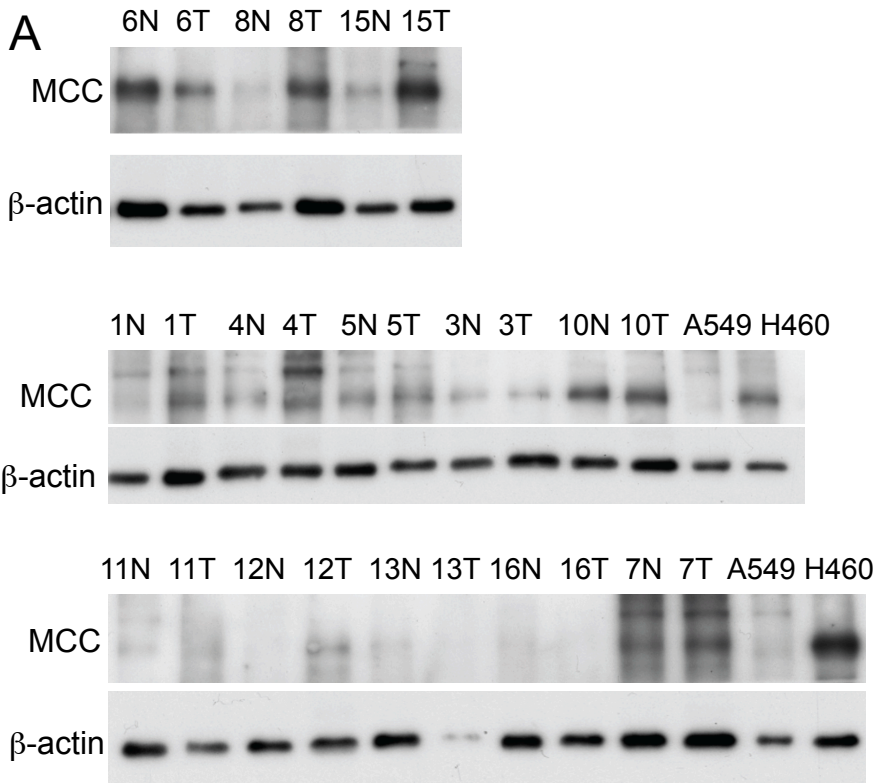
Patient ID	D5S346	INT10	D5S656	MCC met	APC met
58	LOH	LOH	LOH	U	U
54	LOH	NI	LOH	U	M
73	LOH	NI	LOH	U	U
78	LOH	NI	LOH	U	U
221	LOH	NI	LOH	U	U
226	LOH	NI	LOH	U	U
229	LOH	NI	LOH	NR	NR
232	LOH	NI	LOH	U	U
233	LOH	NI	LOH	U	M
59	LOH	No LOH	LOH	U	U
76	LOH	No LOH	NR	U	U
236	No LOH	LOH	LOH	U	U
238	No LOH	LOH	LOH	U	U
60	No LOH	LOH	No LOH	U	U
214	No LOH	NI	NI	NR	NR
50	No LOH	NI	No LOH	U	U
53	No LOH	NI	No LOH	U	U
72	No LOH	NI	No LOH	NR	NR
57	No LOH	NR	No LOH	U	U

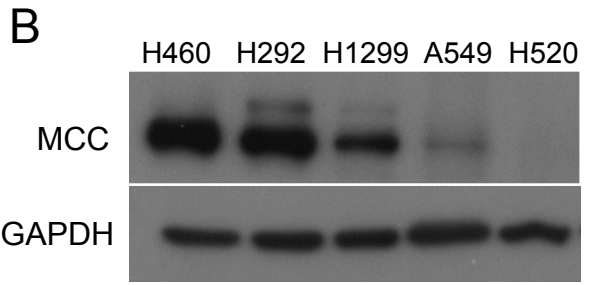
NI=not informative; M=methylation detected; U=no methylation detected; NR=no result

Table 3. Loss of heterozygosity (LOH) and promoter methylation of the MCC and APC genes in large cell carcinomas.

Patient ID	D5S346	INT10	D5S656	MCC met	APC met
79	LOH	LOH	LOH	U	U
130	LOH	LOH	NI	U	M
247	LOH	LOH	NI	U	M
88	LOH	NI	LOH	U	U
253	LOH	NI	LOH	NR	NR
70	LOH	NI	LOH	U	M
67	LOH	NI	NI	U	U
251	LOH	NI	NI	U	U
55	No LOH	LOH	LOH	U	M
56	No LOH	NI	LOH	U	U
213	No LOH	NI	NI	U	U
240	No LOH	NI	NI	U	U
77	No LOH	NI	No LOH	U	U
248	No LOH	No LOH	LOH	U	U
108	No LOH	No LOH	NR	U	U
133	No LOH	NR	No LOH	M	M

NI=not informative; M=methylation detected; U=no methylation detected; NR=no result


A
6N 6T 8N 8T 15N 15T
MCC
β-actin
1N 1T 4N 4T 5N 5T 3N 3T 10N 10T A549 H460
MCC
β-actin
11N 11T 12N 12T 13N 13T 16N 16T 7N 7T A549 H460
MCC
β-actin


B
H460 H292 H1299 A549 H520
MCC
GAPDH

Conflict of Interest

None declared.