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LETTER TO THE EDITOR

# Deregulation of *MYC* and *TP53* through genetic and epigenetic alterations in gallbladder carcinomas

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**Abstract** Gallbladder cancer is a rare malignancy and presents a poor prognosis. MYC and p53 have been implicated in gallbladder carcinogenesis. However, little is known about the molecular mechanisms involved in their regulation in this neoplasia. Here, we evaluated the *MYC* and *TP53* copy numbers in gallbladder tumors and their possible association with protein expression. We also investigated whether *MYC* may be controlled by mutations and DNA promoter methylation. In the present study, 15 samples of invasive gallbladder carcinomas and six control samples were analyzed. On the other hand, the expression of MYC and p53 was more frequent in gallbladder carcinomas than in control samples ($p = 0.002$, $p = 0.046$, respectively). Gain of copies of the *MYC* and *TP53* genes was detected in 86.7 and 50 % of gallbladder carcinomas, respectively. *MYC* and *TP53* amplifications were associated with immunoreactivity of their protein ($p = 0.029$, $p = 0.001$, respectively). *MYC* hypomethylation was only detected in tumoral samples and was associated with its protein expression ($p = 0.029$). MYC mutations were detected in 80 % of tumor samples. The G allele at rs117856857 was associated with the presence of gallbladder tumors ($p = 0.019$) and with MYC expression ($p = 0.044$). Moreover, two tumors presented a pathogenic mutation in *MYC* exon 2 (rs28933407). Our study highlights that the gain of *MYC* and *TP53* copies seems to be a frequent finding in gallbladder cancer. In addition, gain of copies, hypomethylation and point mutations at *MYC* may contribute to overexpression of its protein in this type of cancer.

**Keywords** Gallbladder carcinoma · MYC · TP53 · Genetic alterations · Epigenetic modifications

Dear editor,

Gallbladder cancer is a relatively rare malignancy, but this cancer is the most common biliary tract malignancy with the worst overall prognosis. With the advent of the laparoscope, this disease is now more commonly diagnosed incidentally [1]. However, the etiopathology of this type of tumor remains unclear.

The altered expression of several proteins has been implicated in gallbladder cancer development. MYC is a transcriptional factor that affects up to approximately 15 % of genes in the genomes of many organisms, from flies to humans [2]. MYC plays a role in the regulation of cell

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**Table 1** Clinicopathological characteristics, protein expression, gene copy number, MYC methylation and mutations in gallbladder tissue samples

| Variables | Samples | | |
|---|---|---|---|
| | Controls | Cases | *p* value |
| Gender [N(%)] | | | |
| Female | 5 (83.3) | 10 (66.7) | 0.623 |
| Male | 1 (16.7) | 5 (33.3) | |
| Grade [N(%)] | | | |
| 1 | – | 2 (13.3) | |
| 2 | – | 12 (80) | |
| 3 | | 1 (6.7) | |
| pT stage [N(%)] | | | |
| pT1/pT2 | – | 6 (40) | |
| pT3/pT4 | – | 9 (60) | |
| Stage | | | |
| Early | – | 5 (33.3) | |
| Advanced | – | 10 (66.7) | |
| MYC immunoreactivity [N(%)] | | | |
| Negative | 6 (100) | 3 (20) | 0.002* |
| Positive | 0 (0) | 12 (80) | |
| p53 immunoreactivity [N(%)] | | | |
| Negative | 6 (100) | 7 (46.7) | 0.046* |
| Positive | 0 (0) | 8 (53.3) | |
| *MYC* copies [N(%)] | | | |
| 2 | 6 (100) | 2 (13.3) | 0.001* |
| $\geq 3$ | 0 (0) | 13 (86.7) | |
| *TP53* copies [N(%)][a] | | | |
| 2 | 6 (100) | 7 (50) | 0.051 |
| $\geq 3$ | 0 (0) | 7 (50) | |
| *MYC* promoter methylation [N(%)] | | | |
| Methylated or partial methylated | 6 (100) | 2 (13.3) | 0.001* |
| Hypomethylated | 0 (0) | 13 (86.7) | |
| rs117856857 (C/G) | | | |
| Without G allele | 6 (100) | 6 (40) | 0.019* |
| With G allele | 0 (0) | 9 (60) | |
| rs73707292 (C/G) | | | |
| Without G allele | 6 (100) | 14 (93.3) | 1.000 |
| With G allele | 0 (0) | 1 (6.7) | |
| rs4645949 (C/T) | | | |
| Without T allele | 6 (100) | 11 (73.3) | 0.281 |
| With T allele | 0 (0) | 4 (26.7) | |
| rs28933407 (C/T) | | | |
| Without T allele | 6 (100) | 13 (86.7) | 1.000 |
| With T allele | 0 (0) | 2 (13.3) | |

* $p < 0.05$, by $X^2$-test

[a] One tumoral sample was not evaluated concerning *TP53* copy number

growth and proliferation, metabolism, differentiation, apoptosis and angiogenesis [3]. MYC deregulation was previously reported in gallbladder carcinomas [4–7]. In addition, MYC regulates p53, which plays a key role in regulating the expression of several genes related to DNA repair, cell cycle arrest and apoptosis induction [8]. The loss of function of p53 may induce genetic instability and tumorigenesis. Alterations in p53 are commonly observed in most human cancers, including gallbladder carcinomas [9–11]. However, currently, little is known about the genetic and epigenetic mechanisms involved in MYC and p53 regulation in gallbladder carcinomas.

In the present study, we aimed to evaluate the number of copies of *MYC* and *TP53* in gallbladder tumors and their possible association with the expression of their proteins. Because we observed that MYC immunoreactivity was strongly associated with gallbladder tumors, we also investigated whether this gene might be controlled by mutations and DNA promoter methylation.

During the period from 2008 to 2013, 15 samples of invasive gallbladder carcinomas were obtained in the Hospital João de Barros Barreto (Pará, Brazil). Two samples were advanced squamous cell carcinomas (one with grade 2 and the other with grade 3), and the remaining samples were adenocarcinomas (grade 1 or 2 tumors). All tumors were classified according to TNM staging [12] (Table 1). In addition, 6 gallbladder control samples were obtained from patients submitted to cholecystectomy for the treatment of typical stone disease. The histological examination confirmed the lack of neoplastic cells in the control samples. The tissue samples were formalin fixed and paraffin embedded (FFPE). Sections of FFPE tissue were stained with hematoxylin–eosin for histological evaluation or used for immunohistochemical and molecular analysis. The study was approved by the ethics committee of the Federal University of Pará, Brazil. Written informed consent with the approval of the ethics committee was obtained from all patients prior to the specimen collection.

First, we evaluated the protein expression of MYC and p53 in our samples. Immunohistochemistry (IHC) was performed on FFPE sections with primary mouse monoclonal antibody against MYC (dilution 1:50; sc-40, Santa Cruz Biotechnology, USA, and ZymedH, USA) and p53 (dilution 1:50; DakoCytomation, USA) as previously performed by our group [13–15]. In the present study, the expression of MYC (Fig. 1) and p53 was more frequent in gallbladder carcinomas than in control samples ($p = 0.002$, $p = 0.046$, respectively; Table 1). Although nucleotide sequencing is the most reliable technique to detect gene mutations, immunohistochemical analysis of p53 is

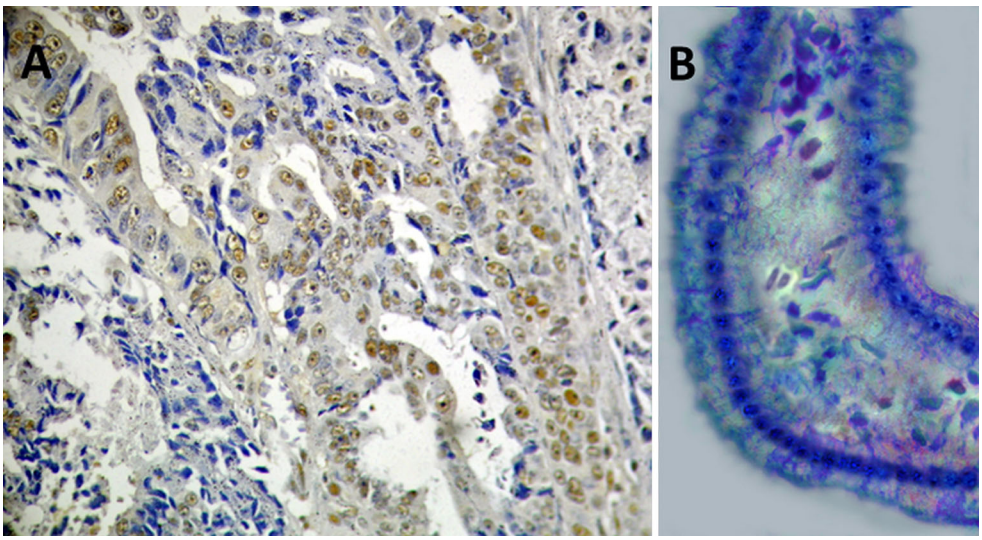


**Fig. 1** MYC immunoreactivity in gallbladder samples (400×). **a** Positive staining in gallbladder carcinoma cells; **b** lack of MYC immunoreactivity in non-cancer gallbladder cells

commonly used as a surrogate for mutational analysis [16]. It has been generally accepted that the wild-type p53 protein has a short half-life, which makes it undetectable by immunohistochemistry. On the other hand, mutant p53 has a much longer half-life and therefore accumulates in the nucleus, creating a stable target for immunohistochemical detection [16]. A previous study demonstrated that p53 protein overexpression correlated well with its gene mutation in gallbladder carcinomas [17]. Therefore, our results are in agreement with possible roles of p53 and MYC in gallbladder carcinogenesis.

Then, we evaluated the number of copies of *MYC* and *TP53* because copy number variation is a frequent finding in several types of cancers and may lead to protein expression deregulation. For this analysis, we performed duplex quantitative PCR (qPCR) using TaqMan CNV assays (Life Technologies, USA) for the target genes (*MYC*: Hs01764918_cn; *TP53*: Hs06423639_cn) and the internal control (*RNase P*: #4403326). Duplex qPCRs were performed in quadruplicate with gDNA as previously performed by our group [18–21]. No loss of gene copies was detected. In the control samples, we did not observe copy number variation. Gain of copies of the *MYC* and *TP53* genes was detected in 86.7 and 50 % of gallbladder carcinomas, respectively (Table 1). Because our qPCR analysis revealed gain of copies of both *MYC* and *TP53*, we cannot exclude the possibility of several chromosome aneuploidies or even polyploidy in gallbladder carcinoma cells. Alterations in chromosome copy number were previously reported in gallbladder carcinomas [7, 22–24].

All samples with *TP53* amplification ($N = 7$) presented immunoreactivity of its protein, and we detected a significant association between these two variables ($p = 0.001$).

**Table 2** MYC immunoreactivity and its gene status in gallbladder carcinomas

| Variables | MYC immunoreactivity | | |
|---|---|---|---|
| | Negative | Positive | *p* value |
| *MYC* copies [N(%)] | | | |
| 2 | 2 (66.7) | 0 (0) | 0.029* |
| ≥ 3 | 1 (33.3) | 12 (100) | |
| *MYC* promoter methylation [N(%)] | | | |
| Methylated or partial methylated | 2 (66.7) | 0 (0) | 0.029* |
| Hypomethylated | 1 (33.3) | 12 (100) | |
| rs117856857 (C/G) | | | |
| Without G allele | 3 (100) | 3 (25) | 0.044* |
| With G allele | 0 (0) | 9 (75) | |
| rs73707292 (C/G) | | | |
| Without G allele | 3 (100) | 11 (91.7) | 1.000 |
| With G allele | 0 (0) | 1 (8.3) | |
| rs4645949 (C/T) | | | |
| Without T allele | 3 (100) | 8 (66.7) | 0.516 |
| With T allele | 0 (0) | 4 (33.3) | |
| rs28933407 (C/T) | | | |
| Without T allele | 2 (66.7) | 11 (91.7) | 0.371 |
| With T allele | 1 (33.3) | 1 (8.3) | |

* $p < 0.05$, by $X^2$-test

However, we cannot exclude that the amplified copies of *TP53* may carry somatic mutations, which may contribute to the stability of the protein.

In addition, *MYC* amplification was detected and associated with its protein expression ($p = 0.029$; Table 2). Only one squamous cell carcinoma and one adenocarcinoma sample did not present a gain of *MYC* copies. To our

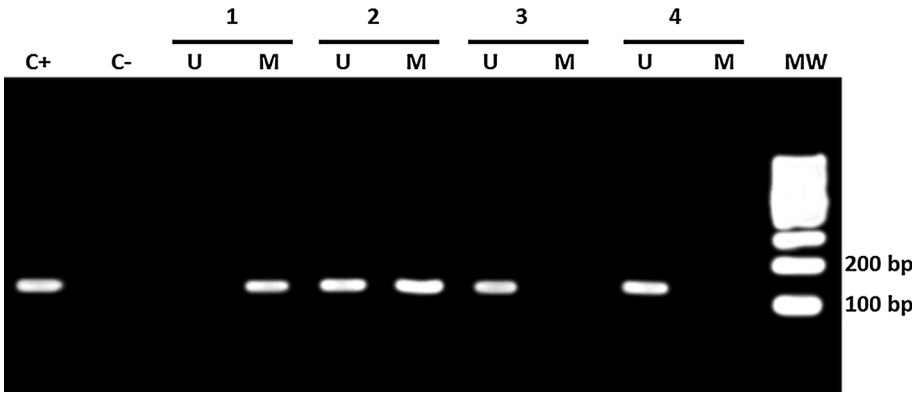


**Fig. 2** Methylation analysis of the *MYC* promoter showing methylated and unmethylated bands. Sample 1 presented a hypermethylated promoter. Sample 2 presented a partially methylated promoter, and Samples 3 and 4 presented hypomethylated promoters. *C*− blank, *C*+ positive control, *gDNA* sample completely methylated, *U* unmethylated, *M* methylated, *MW* molecular weight marker, *bp* base pairs

knowledge, *MYC* amplification in interphase nuclei, shown by FISH analysis, was described in one previous study on gallbladder tumors [7]. In this previous study, only 3.7 % of the tumors presented gene amplification, and approximately 27 % of the cases presented polysomy of chromosome 8, where *MYC* is located. The gain of *MYC* copies, regardless of the number of chromosome 8 copies, has been associated with its overexpression and implicated in the development of other types of cancer [15, 25, 26]. Therefore, gain of copies of *MYC* can contribute to its oncogenic role in gallbladder carcinomas.

Because we had previously reported that *MYC* may be regulated by other genetic and epigenetic alterations [25], we also evaluated *MYC* promoter methylation and the presence of mutations in gallbladder carcinomas and non-tumor samples according to Rosal-Texeira et al. [25]. In the present study, all gallbladder carcinomas and one non-tumor sample presented positive amplification when using an unmethylated primer set. However, *MYC* hypomethylation (positive amplification with only the unmethylated primer set) was only detected in tumoral samples (Table 1; Fig. 2). Therefore, the frequency of *MYC* hypomethylation was significantly higher in tumor samples than in controls ($p = 0.001$, Table 1). In addition, *MYC* hypomethylation was associated with MYC protein expression ($p = 0.029$, Table 2). Interestingly, both tumoral samples without *MYC* hypomethylation and without immunoreactivity of its protein, were classified as squamous cell carcinomas. Hypomethylation at specific promoters can activate the aberrant expression of oncogenes [27]. So far, few studies have discussed the relationship between the methylation pattern of *MYC* and its effect on gene expression and on carcinogenic processes. *MYC* hypomethylation was previously associated with oncogenic progression and metastasis induction in a rat model of liver cancer [28] and in human colorectal [29] and gastric [30–32] cancer samples. To our

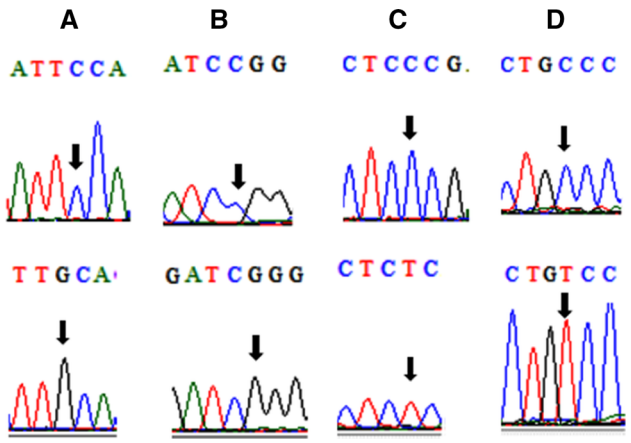


**Fig. 3** DNA sequence chromatograms corresponding to *MYC* common variants (*top*) and rare allele (*bottom*) at **a** rs117856857, **b** rs73707292, **c** rs4645949, **d** rs28933407

knowledge, this is the first study to demonstrate the possible effect of epigenetic modification of *MYC* transcriptional regulation in gallbladder carcinomas. As already proposed for DNA hypermethylation [33], promoter hypomethylation may be used as a new generation of biomarkers.

With regard to *MYC* sequencing, no novel mutation was detected in gallbladder tumors. Twelve (80 %) tumor samples presented at least one known mutation. In total, 16 mutations were identified, with four samples exhibiting co-occurring mutations. No mutation was identified in exon 3. In the 5′ UTR region, five samples presented the genotype GG and four presented the genotype CG at rs117856857 (Fig. 3a); one sample presented genotype GG at rs73707292 (Fig. 3b); and four samples presented genotype CT at rs4645949 (Fig. 3c). The G allele at rs117856857 was associated with the presence of gallbladder tumors ($p = 0.019$, Table 1) and with MYC expression

($p = 0.044$, Table 2). Therefore, the presence of the G allele at rs117856857 may affect MYC expression and contribute to the development of gallbladder tumors.

Concerning missense mutations, two tumors harbored a mutation at codon 72 in exon 2, resulting in a change from proline to serine (rs28933407; Fig. 3d). For rs28933407, one tumor had CT, and the other tumor had TT. This mutation is in an evolutionary conserved sequence of *MYC*: the transactivation domain [3, 34]. In addition, the mutation was considered as most likely damaging according to the PolyPhen software (http://genetics.bwh.harvard.edu/pph2/) and was previously reported as a pathogenic variant in the NCBI dbSNP database (http://omim.org/). Thus, the mutation detected in exon 2 may also affect MYC activity in gallbladder carcinomas. However, we were not able to detect a significant association between the polymorphism rs28933407 and MYC immunoreactivity, most likely due to the low frequency of the T allele.

Interestingly, one squamous cell carcinoma with negative MYC immunoreactivity presented *MYC* gene amplification, and another adenocarcinoma sample without MYC immunoreactivity presented hypomethylation of its gene promoter and was homozygous for the rare allele at rs28933407. Therefore, only one gallbladder carcinoma (squamous cell carcinoma) did not present any MYC or p53 alterations. Further investigations using a large number of samples are still necessary; however, the analysis of genetic and epigenetic mechanisms involved in *MYC* regulation may help in confirming the diagnosis of gallbladder carcinoma. It is important to highlight that immunohistochemistry is a technique that is routinely used in clinical pathology practice; however, standardization of the quantification of expression levels by visual inspection is still a challenge.

To our knowledge, this is the first study to concomitantly describe the genetic and epigenetic alterations of *MYC*, which may have a role in gallbladder carcinomas. However, the main limitation of this study is the limited number of samples. Some statistical analyses exhibit reduced power for detecting significant differences between groups. Therefore, false-negative results may have occurred. In addition, due to the small number of samples, we did not perform multivariate statistical analysis. Although a significant difference ($p = 0.01$, by Mann–Whitney test) between the age of gallbladder carcinoma patients (median $\pm$ interquartile range: 69.5 $\pm$ 15.75) and that of the controls (52 $\pm$ 19.75) was found, the age at the time of surgery and the gender of the individuals were not associated with any other studied variable ($p > 0.05$, for all comparisons).

In conclusion, our study highlights that gain of *MYC* and *TP53* copies seems to be a frequent finding in gallbladder cancer. In addition, gain of copies, DNA promoter hypomethylation and point mutations in the *MYC* gene may contribute to the overexpression of its protein in this type of cancer.

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**Conflict of interest** None.

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