

# Overexpression of the mitotic checkpoint genes *BUB1* and *BUBR1* is associated with genomic complexity in clear cell kidney carcinomas

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**Abstract.** *Background:* A defective mitotic checkpoint has been proposed to contribute to chromosomal instability (CIN). We have previously shown that expression changes of the mitotic arrest deficiency (MAD) gene family plays a role in renal cell cancer (RCC) characterized by numerical chromosomal changes, namely papillary and chromophobe carcinomas, but nothing is known about the expression of mitotic checkpoint genes in the clear cell histotype (ccRCC).

*Methods:* We analyzed the mRNA expression levels of the major mitotic checkpoint genes of the budding uninhibited by ben-zimidazole family (*BUB1*, *BUBR1*, *BUB3*) and of the MAD gene family (*MAD1*, *MAD2L1*, *MAD2L2*) by real-time quantitative PCR in 39 ccRCC and in 36 normal kidney tissue samples. We have additionally analyzed these tumors by comparative genomic hybridization (CGH) in order to evaluate the relationship between mitotic checkpoint defects and the pattern of chromosome changes in this subset of RCC.

*Results:* *BUB1*, *BUBR1*, *MAD1* and *MAD2L1* showed significant expression differences in tumor tissue compared to controls (*BUB1*, *BUBR1* and *MAD2L1* were overexpressed, whereas *MAD1* was underexpressed). Overexpression of *BUB1* and *BUBR1* was significantly correlated with the number of genomic copy number changes ( $p < 0.001$  for both genes) and with Furhman grade of the tumors ( $p = 0.006$  and  $p = 0.005$ , respectively).

*Conclusions:* We conclude that *BUB1* and *BUBR1* overexpression plays a role in cytogenetic and morphologic progression of ccRCC.

Keywords: Clear cell renal cell carcinoma (ccRCC), mitotic checkpoint, gene expression, BUB, MAD

## 1. Introduction

The mitotic spindle checkpoint is a highly conserved mechanism that ensures that sister chromatids are aligned at the metaphase plate and do not separate prior to the bipolar attachment of all duplicated chro-

mosomes, providing equally divided sister chromatids during cell division [14,17,18,34].

Yeast cells defective in the mitotic checkpoint mechanism lose chromosomes at elevated rates and are hypersensitive to mitotic-spindle inhibitors [33,34]. In humans, although mutations in known spindle checkpoint genes are extremely rare [1,2,11–13,20,23,24,35,36,38], several studies have shown evidence that mutations and/or reduced levels of mitotic checkpoint proteins can cause checkpoint malfunction and chromosomal instability (CIN), and thereby contribute to

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tumor formation [10,16,21,33]. A few studies have shown that non-mutational silencing of human *BUB1* and *BUBR1* genes is more common than mutational inactivation, contributing to the development of aneuploidy in human colorectal cancers [25,29].

We have previously evaluated renal cell cancer (RCC) characterized by numerical chromosomal changes, namely papillary and chromophobe carcinomas, for the expression levels of the six major mitotic checkpoint genes and found that expression changes of *MAD1*, *MAD2L1*, and *MAD2L2* play a role in the carcinogenesis of these tumor types [27]. Moreover, the affected genes and the type of expression change (under- or overexpression) differ between the tumor type characterized by multiple monosomies (chromophobe RCC) and the tumor type characterized by multiple trisomies (papillary RCC) [27].

Clear cell renal cell carcinoma (ccRCC) comprises about 75% of all kidney neoplasms [19,31]. It originates from the proximal tubules and presents a defining loss of the short arm of chromosome 3 (3p) in about 98% of the cases, which can occur either by deletion or by an unbalanced translocation between 3p and the long arm of chromosome 5 (70% of the cases) [3]. Other structural and numerical chromosome changes are secondary events in ccRCC, including monosomies (chromosomes 8, 9, 13 and 14), trisomies (chromosomes 12 and 20), and structural changes involving 5q, 6q, 8p, 10q and 14q, which seem to be associated with tumor progression [3,31].

To evaluate the role of mitotic checkpoint defects in cytogenetic progression of ccRCC, we have analyzed the relative transcript levels of *BUB1*, *BUBR1*, *BUB3*, *MAD1*, *MAD2L1* and *MAD2L2*, and compared the findings with the degree of genomic complexity in 39 tumors. To the best of our knowledge, this is the first study evaluating this relationship in ccRCC.

## 2. Materials and methods

### 2.1. Patients and sample collection

From a consecutive series of RCC prospectively collected at the Portuguese Oncology Institute – Porto, Portugal, 39 primary ccRCC diagnosed from 2001 to 2005 were studied. The mean age at diagnosis for these patients was 61 years (range 33–82). The institutional review board approved the project and patients were enrolled in this study after informed consent. For control purposes, non-neoplastic renal tissue distant from RCC (with chromophobe, papillary or clear cell histol-

ogy) was obtained from 36 patients (16 of which included in the present ccRCC series). All patients included in this study underwent radical nephrectomy before any other kind of treatment. Tissue samples intended for genetic analysis were snap-frozen in isopentane immediately after surgery and stored at  $-80^{\circ}\text{C}$ . Five-micron thick sections were cut and stained at every 15 cuts for identification of the areas of carcinoma and normal tissue. The remaining tissues were formalin-fixed and subsequently processed for paraffin embedding. Sections were cut and H&E stained for histopathological evaluation. These procedures comprised the diagnosis, grading and pathological staging (2003 TNM staging system [5]). Five-micron thick sections were cut and stained for identification of the areas of carcinoma and normal tissue.

### 2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from all samples using the FastRNA Green Kit (Qbiogene, Carlsbad, CA, USA) for 45 s, with a speed rating of 6.0 in a FastPrep FP120 Instrument (Qbiogene, Carlsbad, CA, USA). RNA quality was checked on 0.8% agarose gels. Reverse transcription was performed with the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions.

### 2.3. Quantitative RT-PCR

Real-time RT-PCR was performed in 39 ccRCC and in 36 normal controls using a TaqMan<sup>®</sup> ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Primers and probes for the BUB gene family (*BUB1*, *BUBR1* and *BUB3*) and for the mitotic arrest deficiency genes (*MAD1*, *MAD2L1* and *MAD2L2*) were designed using the Primer Express Software (version 2.0; Applied Biosystems, Foster City, CA, USA) [27]. To use as the internal reference gene, commercially available TaqMan<sup>®</sup> reagents with optimized primer and probe concentrations for human hypoxanthine ribosyltransferase (*HPRT*) were purchased from Applied Biosystems (Applied Biosystems, Foster City, CA, USA).

cDNA generated from each sample was split in equal parts for the measurement of the expression of all six mitotic checkpoint genes under study, as well as the endogenous control gene *HPRT*. PCR was performed according to the manufacturer's specifications (Applied Biosystems, Foster City, CA, USA) in sepa-

rate wells for each primer/probe set and each sample was run in triplicate. The 20  $\mu$ l total volume final reaction mixture consisted of 1  $\mu$ M of each primer, 0.25  $\mu$ M of probe, 1  $\mu$ l of TaqMan<sup>®</sup> Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), and 9  $\mu$ l of cDNA (with approximately the same concentration in each case). Negative controls consisted of bidistilled H<sub>2</sub>O. PCR was performed using the following conditions: 50°C for 2 min, 95°C for 10 min, followed by 50 cycles at 95°C for 15 s and 60°C for 1 min. Each plate included multiple non-template controls and serial dilutions of a positive control to construct the standard curve.

To determine the relative mRNA expression levels of each of the six genes in each of the 75 samples, the values of the target gene were normalized against the values of the internal reference gene *HPRT* to obtain a ratio. Thus, expression data of each sample is given as the mean quantity of each gene divided by the mean quantity of the endogenous control *HPRT* gene for the same sample.

#### 2.4. Comparative genomic hybridization

DNA was extracted from frozen tissue samples from the 39 ccRCC according to standard methods. Comparative genomic hybridization was performed as described by Kallioniemi et al. [15], with the modifications previously reported by Ribeiro et al. [28]. To determine the degree of genomic complexity of the tumors (measured by the total number of copy num-

ber changes), loss or gain of entire chromosomes were scored as one change each, whereas partial copy number changes in both chromosome arms and gain and loss in the same arm were scored as separate changes.

#### 2.5. Statistical analysis

For each of the six genes, the normalized expression values of ccRCC were compared with those of normal kidney tissue samples using the non-parametric Mann–Whitney *U* test. The relationship between relative expression changes and the number of chromosome abnormalities was evaluated using Spearman's non-parametric correlation test. Relationship between clinicopathological variables (Furhman grade and pTNM stage) and genetic variables (*BUB1* and *BUBR1* overexpression and the number of chromosome alterations) was assessed using Kruskal–Wallis *H* or Mann–Whitney *U* tests, according to the categorization of the data. To reduce the chance of false-positive findings due to the number of variables tested, the significance level was Bonferroni-corrected to  $p < 0.008$ . All analyses were performed using SPSS version 15.0 (SPSS, Chicago, IL, USA).

### 3. Results

Normalized expression levels for the mitotic checkpoint genes *BUB1*, *BUBR1*, *BUB3*, *MAD1*, *MAD2L1* and *MAD2L2* in normal kidney tissue and ccRCC are presented in Fig. 1. Four out of the six genes

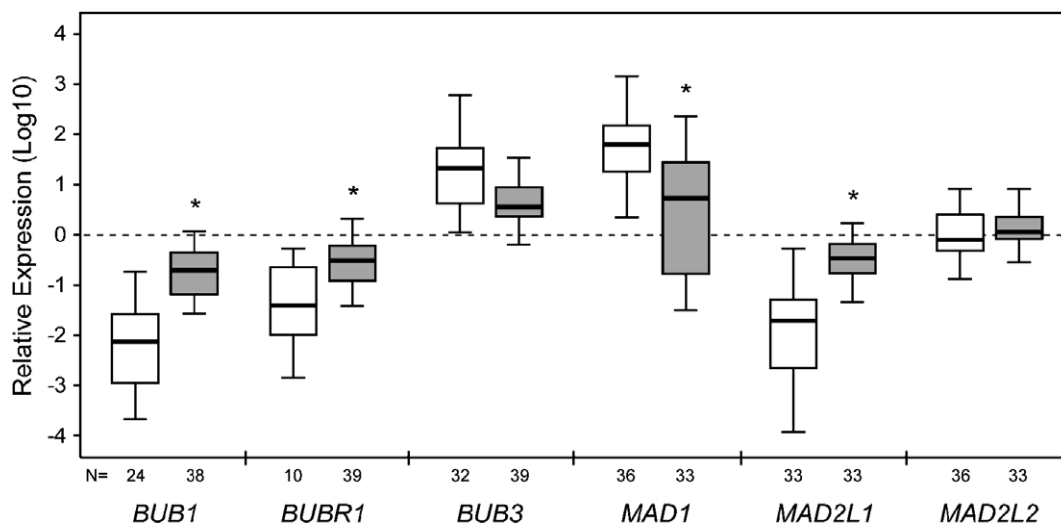


Fig. 1. Distribution of the relative expression levels in normal tissue (white box-plots) and ccRCC (shaded box-plots) for the six genes assessed in this study. \*Statistically significant differences between tumor samples and normal controls (*BUB1*,  $p < 0.001$ ; *BUBR1*,  $p = 0.003$ ; *MAD1*,  $p < 0.001$ ; *MAD2L2*,  $p < 0.001$ ).

showed significant expression differences in tumor tissue compared to normal samples. More specifically, *BUB1*, *BUBR1* and *MAD2L1* were overexpressed ( $p < 0.0001$ ,  $p = 0.0032$  and  $p < 0.0001$ , respectively), whereas *MAD1* was underexpressed ( $p < 0.0001$ ) (Fig. 1). No significant expression changes were detected for *BUB3* or *MAD2L2*.

CGH analysis detected copy number aberrations in 38 of the 39 tumors (97%). The median number of genomic alterations detected was 4 (ranging from 1 to 21 changes). Overexpression of *BUB1* and *BUBR1* was significantly correlated with genomic complexity ( $r_s = 0.632$ ,  $p < 0.001$  and  $r_s = 0.589$ ,  $p < 0.001$ , respectively) (Fig. 2(a) and (b)), but no association was found between specific chromosomal alterations and mitotic checkpoint gene expression. There was a significant correlation between overexpression of *BUB1* and overexpression of *BUBR1* ( $r_s = 0.940$ ,  $p < 0.001$ ) (Fig. 2(c)). Significant correlations were additionally seen between the expression level of several of the re-

maining genes, but with much lower correlation coefficients and without any association with genomic complexity (data not shown). Overexpression of *BUB1* and *BUBR1* was significantly correlated with Fuhrman grade ( $p = 0.006$  and  $p = 0.005$ , respectively), but not with pTNM stage (Table 1). No statistically significant association was found between genomic complexity and Fuhrman grade or pTNM stage.

#### 4. Discussion

Mitotic checkpoint genes have drawn considerable attention for their possible role in CIN in cancer, since a reduction of function for one of these genes can have a profound effect on mitotic stability and maintenance of genomic integrity. We have here demonstrated for the first time that ccRCC has significant differences in the expression of four of six mitotic checkpoint genes when compared to normal kidney tissue, with

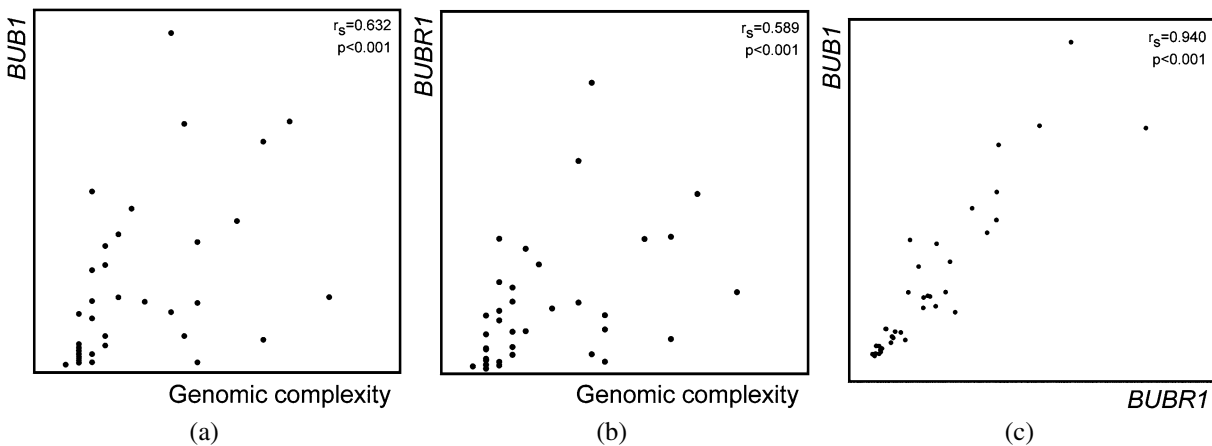


Fig. 2. Correlation between relative expression levels of *BUB1* (a) and *BUBR1* (b) with genomic complexity, and between each other (c). Abbreviations:  $r_s$  – Spearman's correlation coefficient.

Table 1

Association between *BUB1* and *BUBR1* expression levels and chromosomal alterations with Fuhrman grade and pTNM stage

	<i>BUB1</i> expression level	<i>BUBR1</i> expression level	Chromosomal changes
Fuhrman ( <i>n</i> )			
2 (15)	0.06 (0.04–0.13)	0.11 (0.09–0.25)	3 (2–5)
3 (21)	0.24 (0.11–0.45)	0.46 (0.24–0.73)	4 (2.5–9)
4 (3)	0.53 (0.45–0.80)	0.98 (0.32–1.00)	14 (11–16)
	$p = 0.006$	$p = 0.005$	ns
pTNM ( <i>n</i> )			
I + II (33)	0.19 (0.06–0.41)	0.31 (0.10–0.61)	4 (2–10.3)
III + IV (6)	0.20 (0.10–0.87)	0.36 (0.19–1.51)	4 (2–12)
	ns	ns	ns

Notes: Expression levels are given by the median and interquartile range (25–75%). ns – not statistically significant.

*BUB1*, *BUBR1* and *MAD2L1* showing overexpression and *MAD1* being underexpressed (Fig. 1).

CGH analysis revealed that 38 of the 39 tumors (97%) displayed chromosome copy number changes, with a median number of 4 (details on specific genomic changes will be reported elsewhere as part of a larger study). The degree of genomic complexity was significantly correlated with overexpression of *BUB1* and *BUBR1* (Fig. 2(a) and (b)), which points to a significant role of these genes in the development and/or cytogenetic progression of this neoplasia. Interestingly, *BUB1* and *BUBR1* expression changes were not observed in other subtypes of renal cancer, namely papillary and chromophobe carcinomas, where an involvement of the MAD gene family was seen [27]. Overexpression of *BUB1* and/or *BUBR1* has been shown in other tumor types, namely in breast, gastric, bladder, and colorectal carcinomas [8,9,23,29,37,38]. On the other hand both over- and underexpression of these genes have been reported in breast [23] and colorectal tumors [29]. Whereas underexpression was associated with the presence of metastasis and relapse of colon tumors, overexpression was associated with genomic complexity [29], something that is in agreement with our results in ccRCC. The number of chromosome alterations was expected to be significantly associated with Fuhrman grade and pTNM stage, but numbers are too small to conclusively evaluate those relationships.

Upregulation of *BUB1* was significantly correlated with overexpression of *BUBR1* (Fig. 2(c)). The significance of this association is not known. Although these two proteins form a complex at the kinetochores [18,22], they do not recruit each other. Furthermore, *BUB3* is required for kinetochore localization of *BUB1* and *BUBR1* [32], but expression of that gene was not significantly higher in the tumors compared to the normal samples. As far as we know, the positive correlation between overexpression of *BUB1* and *BUBR1* has not been specifically reported before, although overexpression of both genes has been found in other tumor types [8,23,38]. The mechanism behind the combined overexpression remains unclear, as well as its possible causal relationship with increased genomic complexity.

Although overexpression of *BUB1* and *BUBR1* was significantly associated with increased Fuhrman grade (Table 1), only a trend could be seen between overexpression of these genes and tumor stage. This most likely reflects the under-representation of pTNM stages III and IV in our series (only six cases). In other models, increased expression of *BUBR1* characterizes

high-grade breast carcinomas [38] and mRNA levels of *BUB1* were reported to be high in breast cancers with extremely poor outcome [4]. Overexpression of the BUB gene family has been significantly associated with tumor proliferation in gastric cancer [8]. It would be interesting to evaluate in a larger series whether there is any independent prognostic value of *BUB1* and/or *BUBR1* overexpression in ccRCC patients.

The other mitotic checkpoint genes whose expression was significantly altered were *MAD1* (underexpression) and *MAD2L1* (overexpression). We have previously reported underexpression of *MAD1* in chromophobe RCC [27], a tumor characterized by multiple chromosome losses. On the other hand, we have previously shown upregulation of *MAD2L1* in papillary RCC [27], a tumor characterized by multiple chromosome gains. However, the significance of *MAD1* underexpression and *MAD2L1* overexpression in ccRCC is not known, since there was no correlation with genomic complexity. Overexpression of *MAD2L1* has previously been associated with the development of a wide spectrum of tumors in nude mice, as well as with chromosome breaks, anaphase bridges, and whole-chromosome gains/losses in several cell lines [30]. Concomitantly, downregulation of *MAD1* has been found to lead to aneuploidy in human colon carcinomas cell lines [16].

The mechanisms underlying the expression changes we have found are not known. The CGH findings of the 39 ccRCC did not reveal an association between expression levels and copy number changes at a given gene locus. In fact, only one tumor with *BUB1* overexpression had gain of 2q14 and a single case with *MAD2L1* overexpression presented gain of 4q27. Furthermore, no copy number gains were detected at 15q15 (*BUBR1*) and no genomic losses were found at 7p22 (*MAD1*), so mechanisms other than copy number changes must be operative. No alternative mechanisms of upregulation of these three genes have been described [2,6,7,13,23,26,35] and *MAD1* promoter hypermethylation seems to be an uncommon mechanism of expression downregulation in kidney carcinomas or other tumor types [13].

In summary, we found significant expression changes of *BUB1*, *BUBR1*, *MAD1* and *MAD2L1* in ccRCC compared to normal kidney tissue, suggesting a role for these genes in the development of this neoplasia. Moreover, there were significant associations between overexpression of *BUB1* and *BUBR1* with genomic complexity and with Fuhrman grade, implicating its involvement in cytogenetic and morpho-

logic progression in ccRCC. Further studies are warranted to identify the causes, consequences, and clinical relevance of the expression changes we uncovered in ccRCC.

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