CORRELATION BETWEEN REDUCED EXPRESSION OF THE SPINDLE CHECKPOINT PROTEIN BubR1 AND BAD PROGNOSIS IN TONSILLAR CARCINOMAS

Kirsten Hannisdal, MD,1 Espen Burum-Auensen, PhD,3 Aasa Schjølberg,2 Paula M. De Angelis, PhD,2 Ole Petter F. Clausen, PhD2

1 Department of Otorhinolaryngology–Head and Neck Surgery, Oslo University Hospital, Rikshospitalet, Oslo, Norway. E-mail: kirsten@hannisdal.net
2 Division of Pathology and Institute of Pathology, Oslo University Hospital, Rikshospitalet, N-0027 Oslo, Norway
3 Department of Otorhinolaryngology–Head and Neck Surgery, Akershus University Hospital, N-1370 Nordbyhagen, Norway

Accepted 13 November 2009
Published online 9 February 2010 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/hed.21342

Abstract: Background. Spindle checkpoint proteins such as Mad2 and BubR1 are important for chromosome segregation during mitosis. The aim of the present study was to examine their possible impact on prognosis in tonsillar carcinomas and their relation to clinical variables, the prevalence of human papillomavirus (HPV), p53 status, and Ki-67 positivity.

Methods. We examined the expression of Mad2 and BubR1 by immunohistochemistry on tissue microarrays from 105 patients with tonsillar carcinomas.

Results. BubR1 and Mad2 were both expressed in tonsillar carcinomas. Expression of BubR1 was a significant prognostic factor in univariate survival analysis. In multivariate analyses, BubR1 was a significant prognostic factor together with stage, age, and HPV status \( p < .01 \), whereas Mad2 did not show any significant correlations.

Conclusion. We have shown that BubR1 expression is a novel and strong prognostic factor in tonsillar carcinomas, giving additional information to the TNM stage and other known prognostic factors. © 2010 Wiley Periodicals, Inc. Head Neck 32: 1354–1362, 2010

Keywords: tonsillar carcinomas; prognostic factor; spindle checkpoint proteins; BubR1; p53

In tonsillar carcinomas, the most important prognostic factor is the TNM stage. In addition, the increasing prevalence of human papillomavirus (HPV) in tonsillar carcinomas is associated with improved survival.1,2 However, there are individual differences in prognosis independent of TNM status in head and neck cancer. Underlying genetic abnormalities may explain this discordance between the clinical outcome and the TNM status or location.3,4

DNA aneuploidy, such as numerical or structural chromosomal abnormalities, is found in tonsillar carcinomas.1 Chromosomal instability (CIN) implies that cancer cells lose or gain chromosomes or chromosomal material during mitosis,5 representing an increased rate of change in
chromosomal structure that is associated with an increase in bulk DNA, such as aneuploidy. CIN is regarded as 1 of the earliest steps in human carcinogenesis and is also found in head and neck cancer. The mechanisms underlying genomic instability, however, are still unknown. Several reports suggest that aberrant mitotic spindle checkpoint proteins may be an important cofactor in the development of CIN and cancer development. Under normal conditions, the spindle checkpoint ensures a correct distribution of the genomic material to the daughter cells during mitosis. Spindle checkpoint defects and CIN were demonstrated in squamous cell carcinomas of head and neck (SCCHN) in 2003, and these cancers harbor defects in other genomic loci critical in tumor development and spindle checkpoint control, such as p53.

Mad2 and BubR1 constitute 2 of the most important spindle checkpoint proteins and are activated by the lack of microtubule attachments to chromosomal kinetochores during metaphase. Cheung et al reported data suggesting that nasopharyngeal cell lines with downregulation of Mad2 were associated with decreased sensitivity to cisplatin. Similar results are reported for testicular germ cell cancer cell lines and ovarian cancer cell lines. The latter report also showed that the restoration of the spindle checkpoint could be achieved after the induction of Mad2 expression in checkpoint-defective cell lines. The cellular sensitivity to vincristine has also been shown to be associated with high levels of Mad2. Similar results were observed in normal human fibroblasts after the inhibition of BubR1.

The in vivo effect of a faulty spindle checkpoint is drastic to the living organism, as was demonstrated in mice mutant for BubR1 which showed defects in meiotic chromosome segregation resulting in infertility, dwarfism, cataracts, and impaired wound healing. The authors of that paper confirmed earlier studies when reporting that a graded reduction of BubR1 in mouse embryonic fibroblasts led to an increase in the incidence of aneuploidy and in the number of tumors. In an earlier report, we had demonstrated that reduced levels of BubR1 are associated with DNA aneuploidy in sporadic colorectal cancers. Furthermore, cells devoid of BubR1 fail to stop at checkpoints after DNA damage induced by irradiation or doxorubicin, in agreement with the studies of Mad2 showing that normal spindle checkpoint function is necessary for the cellular sensitivity to irradiation and chemotherapy.

We investigated the in vivo expression of Mad2 and BubR1 in tonsillar cancers to elucidate whether these spindle checkpoint proteins could serve as useful prognostic factors in these patients with cancer.

**PATIENTS AND METHODS**

**Patients.** From 1960 until 1996, a total of 310 previously untreated patients with squamous cell carcinoma of the tonsillar region were admitted to the Norwegian Radium Hospital and/or the Department of Otolaryngology, Rikshospitalet. Of the present 105 patients, 60 were diagnosed in the period 1960 through 1984, and 45 during the years 1985 through 1996. The 2 hospitals had a very close clinical cooperation for diagnosis, treatment, and follow-up of these patients, and in this respect they can be regarded as 1 single institution since their merger in 2007. These patients represented mainly the population of southeast Norway with about 1.5 million inhabitants.

Clinical information for all patients such as sex, age, and TNM status was obtained from the medical records. The tumors were retrospectively staged according to the 4th edition (1978) of the Union Internationale Contre le Cancer (UICC) TNM system. The treatment and prognosis have previously been reported in detail for these 310 patients. Complete follow-up and accurate cause of death were obtained by direct patient knowledge, review of outpatient and hospital charts, autopsy findings, direct contact with local hospitals, family physicians, or in some cases by next of kin. The Norwegian Data Inspectorate and the Regional Ethical Committee of South Norway approved the study. Some characteristics of the patient population are shown in Table 1.

**Tumor Material and Histopathologic Evaluation.**

We were able to obtain adequate tumor tissue from original archival tissue blocks of biopsies and surgical specimens from 199 of the 310 patients. Tumor tissue was fixed in formalin and embedded in paraffin. Tissue sections were cut at 4 µm thickness and stained with hematoxylin-eosin and reevaluated by an experienced pathologist (O.P.F.C.). The original diagnosis of squamous cell carcinoma was confirmed for all patients.
Tissue Microarrays and Immunohistochemistry. Tissue specimens fixed in formalin and embedded in paraffin from 105 patients were included in this study. The most representative tumor areas were marked on the tissue block before tissue core sampling using a tissue microarrayer from Beecher (Beecher Instruments, San Prairie, NJ). The diameter of the punching needles was 1.0 mm, and 2 or 3 tissue cores were sampled from each area of interest.

After exposure to 0.5% H$_2$O$_2$ solution, followed by antigen retrieval with Tris-EDTA at pH 9.0, the sections were incubated for 1 hour at room temperature with the primary antibodies against BubR1 and Mad2; (1:150 and 1:50 dilution, respectively), both antibodies were monoclonal and purchased from BD Biosciences (San Jose, CA), product ID numbers: 612503 and 610679, respectively. These antibodies were used based on previous work from this group on the specificity of several antibodies against BubR1 and Mad2. The selected antibodies both showed a high degree of antigen specificity in Western analyses by binding to protein bands of expected molecular weight in protein lysates from tissues shown to have an abundant amount of proteins by immunohistochemistry (HeLa cells, colorectal cancers, and tonsils). Binding was not found in colorectal cancers where the proteins were not detected or in normal colonic mucosa where positive cells were hardly seen. After washing, the slides were incubated for 30 minutes with the HRP-labeled micropolymer conjugated secondary antibody (Envision, DakoCytomation, Via Real Carpenteria, CA). After rinsing, DAB+ (DakoCytoma-

Statistics. The relationships between expression of the various proteins (BubR1, Mad2, Ki-67, and p53), and correlations between protein expressions, clinical data, and HPV status were evaluated by nonparametric methods (Mann–Whitney test, Kruskal–Wallis test, and Spearman’s rho correlation). The median level of expression (% positive cells) of Mad2 and BubR1 was used as a cutoff to discriminate between high expression (above median) and low expression (below or equal to the median). Survival patterns for Mad2 and BubR1 were tested using cutoffs at the quartiles (25%, 50%, and 75%). Survival probabilities were calculated using the Kaplan–Meier product-limit method. Differences between the survival curves were tested by the log-rank test. In the survival analyses, an event was defined as death due to tonsillar carcinoma, fatal complications during treatment, and death from unknown causes. Patients dying of other diseases or other head and neck cancers were treated as censored observations (under
risk until death). The Cox proportional hazards model was used to analyze the simultaneous importance of prognostic factors. The proportional assumption in the Cox model was examined with plots. To fulfill this assumption for TNM stage, stage I and II had to be grouped together and we used the following coding: stage I–II = 0, stage III = 1, stage IV = 2. The Wald

![FIGURE 1. BubR1 expression in percent, histogram (median, 16%).](image1)

![FIGURE 2. Mad2 expression in percent, histogram (median, 27%).](image2)

![FIGURE 3. BubR1 and Mad2 expression in normal (left) and malignant epithelial tissues (right) as visualized by immunohistochemistry using monoclonal antibodies (original magnification ×480).](image3)
test was used for significance testing of the regression coefficients. Any $p$ values < .05 were considered significant.

RESULTS

The distributions of percent tumor cells expressing BubR1 and Mad2 are shown in Figure 1 and Figure 2. The median numbers of positive tumor cells were 16% and 27%, respectively, for BubR1 and Mad2. Figure 3 illustrates BubR1 and Mad2 expression in normal and malignant tonsillar tissues. BubR1 expression is mainly cytoplasmic, whereas Mad 2 expression is mainly nuclear with a distinct nuclear membrane. However, particularly in tumors, Mad 2 expression is also seen in the cytoplasm of some tumor cells, and some BubR1 positive cells also show expression in the nucleus. No significant correlations were seen between BubR1 and Mad2 expression, and no significant relationships between either protein and clinical data, HPV status, or p53 accumulation were found. In Table 2 the distribution of BubR1 expression in HPV-positive and HPV-negative tumors is shown. HPV status was not correlated to levels of BubR1 expression. The BubR1 expression was significantly correlated with Ki-67 positivity ($r = 0.4$, $p < .01$), whereas Mad2 expression was not.

The results for variables tested in univariate survival analyses are shown in Table 3. A significantly reduced survival was found for patients with tumors having low expression of BubR1, with a 5-year survival of 25%, whereas a 5-year survival of 60% was seen for patients with tumors having high BubR1 expression ($p < .01$, Figure 4). Analyzing the survival for BubR1 according to quartiles did not reveal significant trends when comparing the 4 groups. In Table 4 the tests of survival differences between patients with low and high BubR1 expressions within strata for some other variables are presented. The BubR1 expression showed significantly different survival in both age groups, in men, in stage III and stage IV, in HPV-negative, and in p53-positive patients (illustrated for TNM stage III in Figure 5).

In multivariate analyses where BubR1 was tested against known prognostic factors, BubR1 expression was a significant prognostic factor in addition to age, TNM stage, and HPV status ($p < .01$; Table 5). The data on HPV status, p53 accumulation, and Ki-67 positivity are retrieved

![Survival Functions](image)

**FIGURE 4.** Survival by low and high BubR1 expression, $n = 105$ ($p < .01$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

<table>
<thead>
<tr>
<th>Variable</th>
<th>Strata</th>
<th>$n$</th>
<th>$p$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>≤60</td>
<td>45</td>
<td>&lt;.05</td>
</tr>
<tr>
<td></td>
<td>&gt;60</td>
<td>60</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>76</td>
<td>&lt;.01</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>29</td>
<td>0.24</td>
</tr>
<tr>
<td>Period</td>
<td>1960–1985</td>
<td>60</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>1985–1996</td>
<td>45</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>TNM-stage</td>
<td>I–II</td>
<td>17</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>34</td>
<td>&lt;.05</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>54</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>HPV status</td>
<td>Negative</td>
<td>60</td>
<td>&lt;.01</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>42</td>
<td>0.25</td>
</tr>
<tr>
<td>p53 positive</td>
<td>No</td>
<td>27</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>78</td>
<td>&lt;.01</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 2. BubR1 expression and HPV status.</th>
</tr>
</thead>
<tbody>
<tr>
<td>BubR1 expression</td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>Low 33 (55%)</td>
</tr>
<tr>
<td>High 27 (45%)</td>
</tr>
<tr>
<td>Total 60</td>
</tr>
</tbody>
</table>

**Table 3.** Results from univariate survival analyses.

<table>
<thead>
<tr>
<th>Variable</th>
<th>$n$</th>
<th>$p$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (≤60, &gt;60)</td>
<td>105</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>105</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>TNM stage (I–II, III, IV)</td>
<td>105</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>HPV status (−, +)</td>
<td>102</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>BubR1 (≤16, &gt;16)</td>
<td>105</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>p53 positive (no, yes)</td>
<td>105</td>
<td>0.11</td>
</tr>
<tr>
<td>Ki67 (quartiles)</td>
<td>105</td>
<td>0.32</td>
</tr>
</tbody>
</table>
from a larger group (137 patients) and were recently published.25

DISCUSSION

We have shown that BubR1 expression is a significant prognostic factor in tonsillar carcinomas. Low BubR1 expression was associated with poor survival and was a significant prognostic factor both in univariate and multivariate survival analyses. Stratified survival analysis of BubR1 revealed significantly different survival times within TNM stages III and IV, illustrating that BubR1 is a novel, robust, prognostic marker in tonsillar carcinomas in addition to the existing markers. To our knowledge, there are no published reports to date documenting the expression of BubR1 and its prognostic importance in tonsillar carcinomas.

Correlations between BubR1 expression and prognosis have, to date, not been reported for many cancer types. In a series of 104 patients with bladder carcinomas studied by Yamamoto et al.,29 overexpression of BubR1 correlated with higher histologic grade, tumor recurrence, disease progression, and high cell proliferation. Our results also show a significant correlation between BubR1 expression and Ki-67 positivity in tonsillar carcinomas, and a similar association has been previously demonstrated in ulcerative colitis associated,30 and in sporadic colorectal cancers.20 In a recent study of 117 resected pancreatic head adenocarcinomas, BubR1 expression was shown to be an independent, adverse prognostic factor for survival after pancreatoduodenectomy of pancreatobiliary differentiated adenocarcinomas (Gladhaug et al, Histopathology, in press). In 160 patients with ovarian cancer–positive BubR1 expression was associated with shorter recurrence-free survival.31

In the studies of Yamamoto,29 Lee,31 and Gladhaug,32 high expression of BubR1 was associated with adverse survival, which is contrary to observations in the present study. This was unexpected, but there are possible explanations. First, the mechanisms behind BubR1 reduction and thus the ensuing interference with tumor growth may be different in various types of tissues and their respective neoplasms. In our material, the expression of BubR1 seems to be higher than in other reported studies, which may indicate an increased disruption of BubR1 regulation. Furthermore, disruption of BubR1 function may be associated with increased and reduced amount of protein present.

Under normal conditions, BubR1 participates in preventing premature advance from metaphase to anaphase during mitosis, and is activated when the spindle microtubules are not correctly aligned with the chromosomes in metaphase. The Mad2 also participates in this process, and in vitro studies have shown that low levels of Mad2 and BubR1 result in chromosomal instability and premature entrance into anaphase.18,33 In mouse embryonic fibroblasts, BubR1 reduction caused an increase in the incidence of aneuploidy and of tumors.34 Because aneuploidy is associated with bad prognosis, such as in colorectal cancer, low levels of BubR1 might be expected to be associated with poor prognosis, as seen in the present study, due to its documented association with aneuploidy. A recent observation by Burum-Auensen et al20 shows that low level of BubR1 expression is associated with aneuploidy in colorectal adenocarcinomas. However, BubR1 expression was not associated with survival in this study.

Table 5. Results of multivariate survival analyses (n = 102).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Relative death risk</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (&lt;60 = 0, ≥60 = 1)</td>
<td>2.3</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>BubR1 (high = 0, low = 1)</td>
<td>2.0</td>
<td>.01</td>
</tr>
<tr>
<td>TNM stage (I–II = 0, III = 1, IV = 2)</td>
<td>1.8</td>
<td>.001</td>
</tr>
<tr>
<td>HPV (positive = 0, negative = 1)</td>
<td>1.8</td>
<td>.05</td>
</tr>
</tbody>
</table>
DNA ploidy in head and neck carcinomas has been studied in several patient series, and tonsillar carcinomas have previously been shown to display a high frequency of aneuploidy. DNA aneuploidy was more frequently seen in advanced head and neck tumors, and in oral carcinoma lymph node metastases mainly harbored aneuploid tumor clones. In a patient series of 66 cases with tonsillar carcinomas, a high degree of aneuploidy was found in most tumors, and HPV-positive tumors had a lower degree of aneuploidy than HPV-negative tumors with a nonsignificant trend of worse survival associated with aneuploidy. These findings are consistent with an association between low BubR1 expression and aneuploidy also in tonsillar carcinomas, and may explain the poor prognosis in these patients.

Chromosomal changes characterize SCCHN with poor prognosis. Loss on 1q (67 patients), gains on 1q and 16q and losses on 18q (117 patients), losses of 15 and 22 and gains of chromosome 10 and 20 (64 patients) have an impact on survival in SCCHN. In 82 patients with SCCHN, 5 different chromosomal aberrations (amplification of chromosome 11, gain of chromosome 12, and losses of chromosome 5, 6, and 21) were associated with poor prognosis. The BubR1 gene is localized to chromosome region 15q15, and comparative genomic hybridization (CGH) was previously done for 25 cases with tonsillar cancer, and chromosome 15 was lost in 1 tumor and gained in another, suggesting that the observed low BubR1 levels in the present work are not the result of chromosomal loss because the majority of tonsillar carcinomas seem to have a normal copy number of chromosome 15. However, the resolution of CGH is relatively low, and fluorescence in situ hybridization (FISH) is necessary to confirm if the BubR1 gene is lost in tonsillar carcinomas.

The induction of p53 by mitotic checkpoint activation seems to be essential for protecting cells against the abnormal chromosomal ploidization induced by mitotic checkpoint failure. Furthermore, p53 activation in response to mitotic spindle damage requires signaling via BubR1 mediated phosphorylation. This suggests a crosstalk between the mitotic checkpoint and p53. We did not find any correlations between the levels of BubR1 and Mad2 expression, respectively, and p53 accumulation. However, low BubR1 expression was a more apparent prognostic factor within patients with p53 positive tonsillar carcinomas (Table 4). This suggests that p53 dysfunction as indicated by p53 accumulation is of importance for the dismal prognosis of tumors with low BubR1 levels. This is consistent with the documented crosstalk between these 2 proteins for maintaining proper mitotic checkpoint function preventing chromosomal instability.

In this patient series, we recently found a high prevalence of HPV-positive tumors and confirmed previous reports of HPV as a significant prognostic factor in tonsillar carcinomas. Thus, both HPV status and BubR1 expression give additional prognostic information to TNM stage and age. Poor survival for patients with low BubR1 expression was more pronounced in HPV-negative patients, in p53-positive patients, in men, and in TNM stage III–IV (Table 4). However, in all nonsignificant strata, patients with low BubR1 always had the least favorable survival plots. Stratified analyses, as performed here, result in smaller numbers in the subgroups. This may have contributed to false-negative results regarding the survival differences of BubR1 expression within some strata. Later studies should, therefore, test the hypotheses generated in our study regarding different effects of BubR1 within sex, TNM stage, HPV status, and p53 status. However, 1 possible explanation for the difference in survival of patients with tumors with low BubR1 expression within the HPV-negative group may be that the pathogenetic pathways for HPV-positive and negative carcinomas, respectively, are quite different, and that BubR1 interacts differently with these pathways.

We found no prognostic impact of Mad2 in tonsillar carcinomas in this series. Mad2 may indicate worse prognosis in gastric carcinoma. Some data suggest that upregulation of Mad2 correlates with poor prognosis in colon cancer, and interestingly, the homolog spindle checkpoint protein Mad2L2 has recently been shown to predict poor prognosis in colon cancer.

Several studies of the spindle checkpoint and the implications for therapeutic strategies exist. Our results showing low expression of BubR1 to be a strong and novel prognostic factor in tonsillar carcinomas should encourage future studies of the possible therapeutic strategies involving the spindle checkpoint proteins.
REFERENCES


