Association between p53-binding protein 1 expression and genomic instability in oncocytic follicular adenoma of the thyroid

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Abstract. Oncocytic follicular adenomas (FAs) of the thyroid are neoplasms of follicular cell origin that are predominantly composed of large polygonal cells with eosinophilic and granular cytoplasm. However, the pathological characteristics of these tumors are largely unexplored. Both the initiation and progression of cancer can be caused by an accumulation of genetic mutations that can induce genomic instability. Thus, the aim of this study was to evaluate the extent of genomic instability in oncocytic FA. As the presence of p53-binding protein 1 (53BP1) in nuclear foci has been found to reflect DNA double-strand breaks that are triggered by various stresses, the immunofluorescence expression pattern of 53BP-1 was assessed in oncocytic and conventional FA. The association with the degree of DNA copy number aberration (CNA) was also evaluated using array-based comparative genomic hybridization. Data from this study demonstrated increased 53BP1 expression (i.e., “unstable” expression) in nuclear foci of oncocytic FA and a higher incidence of CNAs compared with conventional FA. The association with the degree of DNA copy number aberration (CNA) was also evaluated using array-based comparative genomic hybridization. Data from this study demonstrated increased 53BP1 expression (i.e., “unstable” expression) in nuclear foci of oncocytic FA and a higher incidence of CNAs compared with conventional FA. There was also a particular focus on the amplification of chromosome 1p36 in oncocytic FA, which includes the locus for Tumor protein 73, a member of the p53 family implicated as a factor in the development of malignancies. Further evaluations revealed that unstable 53BP1 expression had a significant positive correlation with the levels of expression of Tumor protein 73. These data suggest a higher level of genomic instability in oncocytic FA compared with conventional FA, and a possible relationship between oncocytic FA and abnormal amplification of Tumor protein 73.

Key words: Thyroid oncocytic tumor, 53BP1, DNA damage response, Genomic instability

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Oncocytic follicular adenomas (FAs), and those that exhibit vascular and/or capsular invasion in the absence of diagnostic nuclear features of papillary carcinomas are diagnosed as oncocytic follicular carcinoma [4-7]. However, the clinical significance of oncocytic change in thyroid tumors remains unclear and controversial. Some studies have indicated that oncocytic carcinomas behave more aggressively compared to the usual variants of well differentiated thyroid cancers, and result in a higher incidence of metastases and a lower survival rate; hence they recommend an aggressive surgical treatment regime for all oncocytic follicular tumors [8-10]. On the other hand, other reports suggest that oncocytic follicular tumors are not more aggressive than their conventional counterparts [11-13].

The DNA damage response (DDR) pathway has emerged as an important factor in tumor suppression. For example, abnormal or defective DDR can result in genomic instability in cells leading to malignancy [14, 15]. The p53-binding protein 1 (53BP1) belongs to a family of evolutionarily conserved DDR proteins with BRCA1 C-terminus domains [16, 17]. 53BP1 is a nuclear protein that rapidly localizes to sites of DNA double-strand breaks and activates p53 along with other kinases, which plays critical roles in DDRs including cell cycle arrest, DNA repair, and apoptosis [18-20]. It has been well documented in vitro by immunofluorescence studies that 53BP1 exhibits faint diffuse nuclear staining in untreated primary cells, whereas after exposure to radiation, 53BP1 is localized at the sites of DNA double-strand breaks and forms discrete nuclear foci [20, 21]. We have recently demonstrated that the presence of 53BP1 nuclear foci as detected by immunofluorescence might serve as a valuable molecular marker of genomic instability during carcinogenesis, which seems to be induced at the precancerous stage during thyroid, skin and cervical carcinogenesis, as follicular adenoma and actinic keratosis [22, 23]. Gorgoulis et al., have also shown, by immunofluorescence study, that hyperplasia of lung and skin was associated with the expression of p53 or 53BP1 nuclear foci [24]. Additionally, it has been suggested that activation of several DDR molecules including 53BP1 occurs in serous tubal intraepithelial carcinoma, which is considered as precursor of the more aggressive high grade serous tubal intraepithelial carcinoma [25]. Our previous study also demonstrated differences in the types of 53BP1 expression between benign and malignant thyroid tumors [22]. The number of discrete nuclear foci of 53BP1 immunoreactivity in follicular cells seems to increase with tumor progression such as that seen in the follicle-adenoma-follicular carcinoma sequence. In addition to exhibiting a punctate staining pattern, intense diffuse nuclear staining of 53BP1 immunoreactivity was also restricted to malignancy [22].

Array comparative genomic hybridization (aCGH) is also a useful technique to detect DNA-copy number aberrations (CNAs) as an indicator of genomic instability and representing chromosomal loss and gain during tumorigenesis; however, this method requires a high quality of DNA in the samples and is rather complicated to perform. A recent study using a transgenic animal model of thyroid neoplasia identified a recurrent gain of chromosomal bands 4D2.3–E2 (43.3%) by aCGH, which is syntonic to a gain of human chromosomal regions 1p36.32–35.3. Tumor protein 73 (TP73) is encoded within 1p36, and belongs to the p53 protein family [26]; dysregulation of the latter plays a critical role in tumorigenesis and significantly affects tumor response to therapy. Significant up-regulation of TP73 transcription, which involves transcription factors that also regulate various vital biological processes including cell differentiation, proliferation, and cell death/apoptosis, has been demonstrated using quantitative reverse transcription-PCR (qRT-PCR) in human papillary thyroid carcinomas. However, analysis of 1p36 and TP73, especially in association with 53BP1, remains unexplored in oncocytic FA.

In an attempt to clarify the potential pathological mechanisms underlying the aggressiveness of oncocytic FA, we analyzed the type of 53BP1 expression using immunofluorescence and determined its association with the degree of DNA-CNAs by aCGH.

Materials and Methods

Thyroid tissues

Twenty-four surgically resected formalin-fixed, paraffin-embedded (FFPE) thyroid tumors including 12 oncocytic and 12 conventional FAs were available for the present study. The pathological diagnoses of all cases were independently confirmed by three pathologists (ZM, MI, and MN). The gender, age, and tumor size of all patients are summarized in Table 1. This study strictly followed the Declaration of Helsinki, and was approved by the Committee for Ethical Issues of Nagasaki University Graduate School of Biomedical Sciences (Protocol No. 15062617). As this was a ret-
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unstable; three or more discrete nuclear foci and intense heterogeneous nuclear staining. The number of tumor cells expressing each type of 3BP1 immunoreactivity in each viewing area was counted, and the percentage of positive cells was calculated in each case. Finally, the type of 3BP1 expression pattern in each case was determined by the predominant expression pattern.

Immunofluorescence analysis of 3BP1 expression

After antigen retrieval with microwave treatment in citrate buffer, deparaffinized 4 μm sections were preincubated with 10% normal goat serum. Tissue sections were then reacted with an anti-3BP1 rabbit polyclonal antibody (Bethyl Laboratories, Montgomery, TX, USA) at a 1:200 dilution for 1 hour at room temperature. The slides were subsequently incubated with an Alexa Fluor 488-conjugated goat anti-rabbit antibody (Invitrogen, Carlsbad, CA, USA). Specimens were counterstained with 4’,6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Vysis Inc., Downers Grove, IL, USA), analyzed, and photographed using a High Standard All-in-One Fluorescence Microscope (Biorevo BZ-9000; KEYENCE Japan, Osaka, Japan). Signals were analyzed at ×1,000 magnification. Based on our previous reports [22, 23], we categorized 3BP1 immunoreactivity into three types in the current study as shown in Fig. 1: i) stable: absent or faint nuclear staining; ii) intermediate: one or two discrete nuclear foci; or iii) unstable; three or more discrete nuclear foci and intense heterogeneous nuclear staining.* assessed by Mann-Whitney U test; **: assessed by Fisher’s exact test.

Table 1  Comparisons of clinicopathologic data and type of 3BP1 expression in oncocytic and conventional follicular adenomas

<table>
<thead>
<tr>
<th>Type</th>
<th>Sex M/F</th>
<th>Age [years ± SD]</th>
<th>Size [cm²]</th>
<th>Type of 3BP1 expression</th>
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<tr>
<td></td>
<td></td>
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<td>Stable</td>
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<tr>
<td>Conventional</td>
<td>2/10</td>
<td>47.8 ± 17.3</td>
<td>5.4 ± 2.9</td>
<td>9 (75%)</td>
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<td>Oncocytic</td>
<td>1/11</td>
<td>64.7 ± 20.1</td>
<td>6.1 ± 3.9</td>
<td>1 (8.3%)</td>
</tr>
<tr>
<td>p-value</td>
<td>0.03*</td>
<td>0.6*</td>
<td></td>
<td>0.0028**</td>
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Type of 3BP1 expression: Stable type: absent or faint nuclear staining; Intermediate: ≤2 nuclear foci; Unstable: ≥3 nuclear foci or intense and heterogeneous nuclear staining.* assessed by Mann-Whitney U test; **: assessed by Fisher’s exact test.

DNA extraction

Genomic DNA was extracted from tumor and normal areas in FFPE tissues as reported previously [22]. Tumor areas containing more than 70% tumor cells as identified by a guide slide stained with hematoxylin and eosin were microdissected from each FFPE block using 10 × 10 μm-thick-sections, and transferred into tubes. Paraffin removal was performed in 80% xylene; then tissues were washed twice with absolute ethanol and deparaffinized tissue pieces were spun down at 15,000 × g for 10 minutes. After drying, pellets were resuspended in 360 μl buffer ATL (QIAamp DNA Mini Kit, Qiagen, Hilden, Germany) and incubated at 95°C for 15 minutes, followed by cooling to room temperature. Samples were immediately digested with proteinase K for 72 hours at 56°C in a rotation oven with periodic mixing and addition of fresh proteinase K every 24 hours. DNA was collected using the QIAamp DNA Mini Kit according to the manufacturer’s instructions. Extracted DNA was quantified on a NanoDrop.

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Fig. 1  Representative images of 3BP1 staining patterns: stable type; intermediate type; and unstable type.
heated by microwave in 0.01 M citrate buffer (pH 6.0) and pretreated with 0.3% pepsin. Subsequently, slides were immersed in 0.1% NP-40 and denatured by heating in 70% formamide/2× SSC. The probe mixture was also denatured and applied to the pretreated tissues. The slides were incubated for 16 hours at 37°C in a humidified chamber. After hybridization, slides were washed, counterstained with DAPI (Vysis Inc.), and photographed. Signals were analyzed in 10 viewing areas per case at ×1,000 magnification.

Immunohistochemical analysis of TP73 expression

An immunohistochemistry study was performed to determine the level of TP73 expression in the patient samples. After antigen retrieval by heating the tissue sections in a microwave for 20 minutes in citrate buffer (pH 6.0), the sections were immersed in 0.3 % H2O2 solution for 30 minutes to block endogenous peroxidase activity and incubated for 1 hour at room temperature with an anti-p73 rabbit monoclonal antibody (Abcam, Tokyo, Japan) at a 1:50 dilution in a humidified chamber. To detect the immunostaining, Histofine Simple Stain™ MAX PO (MULTI) (Nichirei Biosciences Inc., Tokyo, Japan) was used according to the manufacturer’s instruction. For evaluation of TP73 expression, we counted the number of positively stained cells in 10 fields at ×400 magnification per section and calculated the mean percentage of positive cells, which was defined as TP73 immunoreactivity in each case. The level of TP73 immunoreactivity was categorized into four groups according to the percentage of positive cells as follows: 1) negative: 0<5%; 2) low: 5<30%; 3) moderate: 30<60%, or 4) high: ≥60%.

Statistical analysis

Comparisons of age, gender, and tumor size between the patients with oncocytic and conventional FA were carried out using the Mann-Whitney U test. Associations between the type of 53BP1 expression (stable, intermediate, or unstable) and histologic type (oncocytic or conventional FA) were assessed by the Fisher’s exact test. The Cochran-Armitage test was used to compare the level of TP73 immunoreactivity between histologic types. The level of TP73 immunoreactivity was categorized into four groups according to the percentage of positive cells as follows: 1) negative: 0<5%; 2) low: 5<30%; 3) moderate: 30<60%, or 4) high: ≥60%.
activity in FA were evaluated by Pearson’s correlation analysis. The PHREG procedure in SAS software (version 8.2; SAS Institute, Cary, NC, USA) was used for calculations. All tests were one-tailed, and a p-value <0.05 was considered statistically significant.

**Results**

**Type of 53BP1 expression and its association with CNA as assessed by aCGH**

Baseline patient data are shown in Table 1. Representative images of 53BP1 expression in both oncocyic and conventional FA by immunofluorescence analysis are depicted in Fig. 2. Relative to the stable type of 53BP1 expression, the unstable pattern has been shown to increase during carcinogenesis [22]. Notably, in the current study, the incidence of unstable 53BP1 expression was significantly higher in oncocyic FA than in conventional FA as assessed by the Fisher’s exact test (p=0.0028) as shown in Table 1. Next, we further examined the association between the type of 53BP1 expression and genomic instability in our series. The degree of CNA was measured by aCGH in the samples that met the DNA quality standard for this assessment, which consisted of 4 of the 12 oncocyic FA cases showing unstable 53BP1 expression and 4 of the 12 conventional FA cases showing stable or intermediate 53BP1 expression. These results are shown in Supplementary Fig. 1. Statistical analysis using the Student’s t-test revealed that the total length of the CNAs was significantly longer in oncocyic FA than in conventional FA [mean 884.3 Mbp (range 466–1526) vs. mean 84.5 Mbp (range 21–239), p=0.035], and the total number of CNAs was also significantly

![Fig. 2](image_url)

**Fig. 2** Hematoxylin and eosin (HE) staining and 53BP1 expression in oncocyic and conventional FA. Left and right upper panels show H&E staining (original magnification: ×400). Immunofluorescence images of 53BP1 expression in oncocyic (left lower panel) and conventional (right lower panel) follicular adenomas. Left lower panel: diffuse intense and heterogeneous nuclear staining with rare nuclear foci (unstable/intermediate type) (original magnification: ×1,000). Right lower part: faint nuclear staining (stable type) (original magnification: ×1,000). The scale bars indicate 10 μm.
larger in oncocytic FA than in conventional FA [mean 109.5 genes (range 21–179) vs. mean 20.5 genes (range 3–54), \( p = 0.0432 \)].

**Amplification of chromosome 1p36 in oncocytic FA**

Based on the aCGH results, we further looked into the specific chromosomal locations wherein DNA amplification occurred. Notably, analysis demonstrated amplification of chromosome 1p36 in 3 of 4 oncocytic FA cases, but not in 4 conventional FA cases (Fig. 3). To verify this finding, we assessed the amplification of chromosome 1p36 by FISH (Supplementary Fig. 2). FISH analyses correctly demonstrated the presence of 1p36 gene amplification in all three oncocytic FA cases, which corroborated the finding of 1p36 amplification by the aCGH method. These results suggested that amplification of 1p36 might be one of the defining genomic features of oncocytic FA. These data led us to focus on the tumor protein TP73 in 1p36, which has been shown to be frequently dysregulated during carcinogenesis in various malignancies [28, 29]. Representative images of TP73 expression in both oncocytic and conventional FA obtained using immunohistochemistry are depicted in Fig. 4. Our work demonstrated that the level of TP73 immunoreactivity was substantially higher in oncocytic than in conventional FA (\( p = 0.0001 \)).

Importantly, we found a significant positive correlation (\( r = 0.5983, \ p = 0.002 \)) between the percentage of tumor cells exhibiting unstable 53BP1 expression and the percentage of cells positive for TP73 expression in oncocytic FA as evaluated using Pearson’s correlation analysis (Fig. 5). Taken together, these data indicated that oncocytic FA demonstrates an elevated TP73 protein level that correlates with the occurrence of DNA double strand breaks.

**Discussion**

The present study demonstrates for the first time the existence of differences in the type of 53BP1 expression between oncocytic and conventional FA. The prevalence of unstable 53BP1 immunoreactivity, which is suggestive of the induction of endogenous DDR mechanisms, was significantly higher in oncocytic than conventional FA, indicating a higher level of genomic instability in oncocytic FA. This study also revealed a higher incidence of CNA in the tumor DNA of oncocytic FA exhibiting unstable 53BP1 expression, providing further evidence for a role of genomic instability during oncocytic FA tumorigenesis and its association with the pattern of 53BP1 expression. Previous CGH analyses found that chromosomal aberrations were common in oncocytic FA/Hürthle cell adenoma [30-32]. Although some studies have found that carcinomas have more chromosomal gains and losses than adenomas, in others the differences were not statistically significant [30-32]. In contrast, Dittori et al. demonstrated that the diffuse accumulation of mitochondria in thyroid follicular lesions with oncocytic features was specifically associated with aneuploidy, indicating numerical chromosomal changes [33], suggesting the existence of an association between genomic instability and oncocytic morphology regardless of histological type such as hyperplastic nodule, adenoma, or carcinoma.

It is unclear why oncocytic FAs exhibit increased genome instability compared to conventional FAs. We note that patients with oncocytic FA were older than those with conventional FA, consistent with previous findings regarding the general population of patients with oncocytic FA [34]. Although we cannot completely exclude the possibility that age difference contributed to the differential expression of 53BP1, our previous studies indicated that 53BP1 expression pattern is largely influenced by pathological grade and the pattern of neoplasms [22, 23, 27, 35]. Oncocytic cells contain large numbers of mitochondria harboring morphologic, functional, and genetic abnormalities. The identification of point mutations and large deletions of mitochondrial DNA [3] and of mutations in gene-associated retinoid-interferon-induced mortality-19 (GRIM-19), a nuclear gene involved in the mitochondrial respiratory chain and cell death, appears to be specific for oncocytic tumors regardless of their histotype (FA, or follicular or papillary carcinoma) [3]. It has been suggested that these deletions or mitochondria DNA changes might lead to a progressive increase in the percentage of abnormal mitochondria [3]. Thus, it could be speculated that mitochondrial dysfunction might block the apoptotic process, resulting in an increase in the survival of genetically injured cells and, simultaneously, genomic instability during tumorigenesis in oncocytic neoplasms. Further study is required to elucidate the presiding mechanisms underlying increased DNA double strand breaks in oncocytic FAs.

Our aCGH analysis demonstrated the amplification of chromosome 1p36 in oncocytic FA tumors showing unstable 53BP1 expression, but not in conven-
Fig. 3 Increased genomic aberrations in oncocytic follicular adenoma (FA). Graphic display of genomic aberrations in oncocytic FAs (left side) and conventional FAs (right side) in Chromosome 1q as assessed by array comparative genomic hybridization. Amplification of the 1q36 area, marked in upper section by the long red line specifying location where have happened most amplification or deletion, is observed in oncocytic FA.
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Amplification of chromosome 1p36 in oncocytic FA was additionally confirmed by FISH. This is consistent with the report by Wada et al., who also found a gain of chromosome 1p36 in oncocytic FA by CGH analysis [32]. In the current study, we further demonstrated a significant positive correlation between the percentage of tumor cells in FA expressing unstable 53BP1 expression and the percentage of cells positive for the expression of TP73, which is encoded by a gene located on chromosome 1p36.2-3. Previous studies have also suggested an impact of TP73 gene alteration on human thyroid tumorigenesis [36]. The TP73 gene has been shown to encode a large variety of diverse transcripts that are regulated by extensive alternative splicing [37]. These transcripts can be generally categorized into two main groups, encoding transcriptionally active and N-terminally truncated (ΔN) isoforms [37]. ΔNp73 plays a dominant-negative role in inhibiting the transcriptional and other biological activities of the transcriptionally active isoforms, which are linked

Fig. 4  Comparison of 53BP1 expression patterns and TP73 immunoreactivities between oncocytic FA (A-C) and conventional FA (D-F). H&E staining of oncocytic and conventional FA (A, D). A strong TP73 immunoreactivity (C) is observed in oncocytic FA showing unstable 53BP1 expression (B), while TP73 immunostaining (F) is faint in conventional FA with intermediate 53BP1 expression (E).

Fig. 5  Positive correlation between unstable 53BP1 expression and TP73 immunoreactivity in follicular adenoma (r = 0.59832, p = 0.0020, by Pearson’s correlation analysis).
to cancer development [38]. Accordingly, ΔNp73 is upregulated in many human cancers including liver, ovarian, breast, and melanoma [28, 29, 39-41]. The correlation of 53Bp1 nuclear expression pattern and TP73 does not explicitly indicate that unstable 53BP1 expression underlies the aberrant TP73 expression. However, it confirms that increased expression of 53BP1 is associated with other signs of genomic instability as demonstrated by array CGH. It has recently been reported that oncocytic follicular carcinomas also have more aggressive features, such as higher rate of lymphovascular invasion, compared to conventional types [42], suggesting that they might also carry elevated genomic instability. However, whether 53BP1 nuclear foci are increased in oncocytic follicular carcinomas is yet to be elucidated and requires further examination.

In summary, this study demonstrated the unstable pattern of 53BP1 expression in oncocytic FA and its association with a higher incidence of CNA as assessed by aCGH. Although further studies are required to determine the pathological and clinical roles of 53BP1 nuclear foci in follicular cell-derived neoplasms, the results of the current study suggest that oncocytic FA exhibits elevated genomic instability compared to non-oncocytic FAs.

**Acknowledgments**

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**Disclosure Statement**

The authors disclose no financial or personal relationships with other people or organizations that could inappropriately influence this work.

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**Supplementary Fig. 1** Graphic display of whole genomic aberrations in oncocytic follicular adenomas (A) and conventional follicular adenomas (B). Red bars in the panel indicate gain of DNA copy number and green bars indicate losses of the DNA copy number.
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Supplementary Fig. 2 Dual-color interphase fluorescence in situ hybridization (FISH) analysis using a commercially available Vysis 1p36 (labeled with red color)/1q25 (labeled with green color) probe set. In both conventional FA (A) and in the non-tumor area surrounding oncocytic FA (B), the follicular cells show two red signals and two green signals, suggesting the presence of a wild-type (unamplified) 1p36 region on each of the 2 chromosomes (original magnification: ×1,000).


