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Disruption of chromosomal locus 1p36 differentially modulates TAp73 and Np73 expression in follicular lymphoma

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Abstract

The *TP73* gene is located at the chromosome 1p36 locus that is commonly disrupted or deleted in follicular lymphoma (FL) with poor prognosis. Therefore, we analyzed the expression of the pro-apoptotic TAp73 and anti-apoptotic Np73 isoforms in FL cases with normal or abnormal 1p36. We observed a significant increase in Np73 expression and Np73:TAp73 ratio, lower expression of cleaved caspase-3 and a higher frequency of Ki-67 and PCNA positive cells in FL cases with abnormal 1p36. A negative correlation between the Np73:TAp73 ratio and cleaved caspase-3 expression, and a positive correlation between Np73 expression and Ki-67 or PCNA were observed. The expression of TAp73 and its pro-apoptotic transcriptional targets Bim, Puma, and Noxa were significantly lower in FL compared to reactive follicular hyperplasia. Together, our data demonstrates that 1p36 disruption is associated with increased Np73 expression, decreased apoptosis and increased proliferation in FL.

Keywords

chromosome 1p36; p73 isoforms; follicular lymphoma

Introduction

Non-Hodgkin lymphoma (NHL) is a heterogeneous group of lymphoid neoplasms with distinctive morphologic, immunologic, cytogenetic, and molecular features. Follicular lymphoma (FL) is the second most common type of NHL and constitutes about 70% of the indolent lymphomas (1). Structural chromosome aberrations play a critical role in the pathogenesis of FL. Following the hallmark t(14;18)(q32;q21) translocation, the pathogenesis of FL involves the sequential accumulation of secondary genetic events. These

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secondary genetic events may influence the clinical manifestations and progression of the disease (2–4).

Rearrangement of the chromosome 1p36 locus is frequent in FL and is associated with a poor prognosis (5;6). However, the molecular events mediating this outcome are poorly understood. Of the genes mapped to this locus, *TP73* belongs to the *TP53* family of tumor suppressor genes which are critical in regulation of the cell cycle and apoptosis. There is a high degree of homology between *TP73* and *TP53*, which enables p73 to transactivate p53 target genes (7–9). The *TP73* gene locus encodes two types of isoforms due to alternate promoter usage and differential mRNA splicing. TAp73 isoforms (containing the transactivation domain) are tumor suppressive, whereas Np73 isoforms (truncated and lacking the transactivation domain) are oncogenic by antagonizing both TAp73 and p53 (7–9). The balance between TAp73 and Np73 isoforms and their harmony with other members of the *TP53* family determines the net cellular responses (7–9).

The *TP73* locus is commonly deleted in NHL (5;10). The expression of *TP73* is variable in normal and tumor tissues, and the role of *TP73* in tumor progression is not well established. *TP73* gene expression is observed in normal lymphocytes but is decreased in NHL(11). Some studies have reported transcriptional silencing of *TP73* in NHL by DNA methylation (reviewed in (11)). Loss of heterozygosity and decreased expression of *TP73* is related to tumor aggressiveness in breast cancer (7), but associated with a favorable prognosis in hepatocellular carcinoma (12). However, the role of p73 isoforms in the biology of FL is unknown.

In this study, we analyzed the levels of TAp73 and Np73 isoforms (mRNA and protein) in FL biopsies with or without a chromosome 1p36 abnormality, and determined the functional significance. Our results indicate for the first time that 1p36 abnormalities differentially modulate p73 isoform expression in FL with increased Np73 expression and a high Np73:TAp73 ratio, resulting in decreased apoptosis and increased proliferation of the tumor cells.

Material and Methods

Tumor specimens

Diagnostic biopsies (n = 20) of low grade FL, which were cytogenetically analyzed at the University of Nebraska Medical Center, were used for this study. In addition, lymph node biopsies (n = 5) obtained from patients with reactive follicular hyperplasia (FH) were used for comparison. The study was approved by the Institutional Review Board of the University of Nebraska Medical Center.

Cytogenetic characterization and fluorescence in situ hybridization (FISH) procedures

Chromosome preparations were obtained from diagnostic biopsies of FL (n = 11) following a protocol described previously (5). Briefly, mechanically desegregated cells were cultured for 24 and 48 hours at 37°C in RPMI media with 20% fetal bovine serum and antibiotics. The cells were exposed to colcemid (0.05 µg/ml; Invitrogen, Grand Island, NY) for approximately 40 minutes before initiation of harvest. Following hypotonic treatment

(0.074M KCL solution for 20 minutes at 37°C), the cells were fixed in freshly-prepared fixative (3:1 methanol:glacial acetic acid). After three washes, air dried slides were incubated at 60°C overnight and Giemsa banding using Wright's stain was performed. A minimum of 20 metaphases were analyzed. Karyotypes were described according to the International System for Human Cytogenetic Nomenclature (13).

For validation of 1p36 disruption, direct-labeled, locus-specific probes for 1p36 that includes the *TP73* locus, and a control locus on 1q25, were used (Abbott/Vysis, Inc. Abbott Park, IL). FISH was performed by co-denaturation on a HYBrite® instrument (Abbott/Vysis, Inc. Abbott Park, IL) at a denaturation temperature 75°C for 1 minute, followed by overnight hybridization at 37°C. The slides were then washed with 0.4XSSC/0.3% NP-40 at 72°C for 2 minutes. The cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI). At least 100 interphase nuclei were examined on an Olympus BX51 microscope equipped with appropriate filters and imaged with the Cytovision Image Analysis System (Applied Imaging, Santa Clara, CA).

mRNA analysis

Total RNA was isolated from the diagnostic biopsies (n = 20) using the RNeasy Mini kit (Qiagen Inc, Valencia, CA) and quantified spectrophotometrically using the NanoDrop 2000 (Nanodrop, Wilmington, DE). For quantitative real-time RT-PCR analysis, first strand cDNA was generated using oligo (dT)₁₈ (Fermentas, Glen Burnie, MD) and Superscript II RT (Invitrogen, Grand Island, NY). Two µL of the resulting cDNA was used in the PCR reactions using the following primer sets: TAp73 Forward 5'-GCA CCA CGT TTGA GCA CCT CT-3'; Reverse 5'-GCA GAT TGA ACT GGG CCA TGA-3'; Np73 Forward 5'-CAA ACG GCC CGC ATG TTC CC-3'; Reverse 5'-TGA ACT GGG CCG TGG CGAG-3'; β-Actin Forward 5'-TGA AGT GTG ACG TGG ACA TC-3'; Reverse 5'-ACT CGT CAT ACT CCT GCT TG-3'. The primer set for TAp73 was designed to amplify all of the isoforms containing the transactivation domain. For Np73, the forward primer was designed to detect a sequence unique to the Np73 transcript 5' untranslated region of exon 3 (7). The primer sets for p73 pro-apoptotic targets were: Bim Forward 5'-TGG CAA AGC AAC CTT CTG ATG-3'; Reverse: 5'-CAG GCT GCA ATT GTC TAC CT-3'; Puma Forward 5'-GGA GGG TCC TGT ACA ATC TC-3'; Reverse: 5'-GCT ACA TGG TGC AGA GAA AG-3'; and Noxa Forward 5'-GCT GGA AGT CGA GTG TGC TA-3'; Reverse: 5'-CCT GAG CAG AAG AGT TTG GA-3'. The housekeeping β-actin gene was used to normalize gene expression. For all primer sets, the specificity of the amplified product was confirmed using both melting curve analysis and agarose gel electrophoresis. Annealing temperature for each primer set was optimized by performing gradient PCR. All samples were evaluated in duplicate to ensure consistency of the results. Quantitative real-time PCR reactions were carried out using FastStart Sybr Green Master Mix (Roche, Indianapolis, IN) and a MyIQ iCycler (Bio-Rad, Hercules, CA). The *ct* value was normalized using β-actin for relative gene expression analysis. For calculation of the relative expression, *ct* was used (14).

Immunohistochemistry

Immunohistochemical (IHC) analysis was performed on a tissue array prepared from diagnostic biopsies of FL with (n = 5) or without a (n = 5) 1p36 abnormality, or FH (n = 5). Three representative cores of tissue from each case were investigated. The following antibodies were diluted in antibody diluent (Pharminogen, San Diego, CA) in the following ratios: anti-p73 (32-4200; Invitrogen, Grand Island, NY) which reacts with the full length human p73 (1:300); anti- Np73 (IMG-313A; Imgenex, San Diego, CA), (1:1000); anti-Ki67 (H-300; Santa Cruz, Dallas, TX), (1:70); anti-PCNA (PC-10; Santa Cruz, Dallas, TX), (1:40); and anti-cleaved caspase-3 (ASP175 5A1E; Cell Signaling Technology, Danvers, MA), (1:200). Biotinylated secondary antibody, either anti-mouse (BA-2000; Vector Laboratories, Burlingame, CA) or anti-rabbit (BA-1000; Vector Laboratories, Burlingame, CA), diluted 1:500 in PBS and incubated for 60 min was used. Immunohistochemical evaluation was done microscopically (Nikon; Melville, NY) using a 5 × 5 reticle grid (Klarmann Rulings, Litchfield, NH). In order to evaluate the specificity of staining, a negative control serial section from the same tissue array was processed through the same procedure but omitting the secondary antibody step. The immunostaining was evaluated using a scale of 1 to 4 as follows: 1 (less than 25% of the cells are reactive), 2 (25–50% of the cells are reactive), 3 (51–75% of the cells are reactive), and 4 (greater than 75% of the cells are reactive). WCIF ImageJ software (NIH) was used to facilitate manual counting of Ki-67, PCNA, and caspase-3 positive cells. The percentage of positive cells in three random high-power fields (HPFs) was calculated and the average of the three HPFs was used for further analysis.

Statistical analysis

All values in the figures are expressed as mean ± SEM. We used Mann-Whitney test (two-tailed) for comparisons between cases with or without 1p36 abnormalities and FH samples. For correlation analysis, non-parametric bivariate correlation analyses (Kendall's tau_b and Spearman's rho) were used (two-tailed). SPSS software was utilized for performing statistical analysis (SPSS; Chicago, IL).

Results

1p36 chromosomal abnormalities modulate p73 expression

For p73 expression studies, RNA from ten cytogenetically-characterized FL cases with 1p36 abnormalities and ten cases without 1p36 abnormality was utilized. The status of each 1p36 abnormality was confirmed by FISH analysis (Figure 1). Real-time quantitative PCR was used to compare the expression of TAp73 and Np73 between cases with or without 1p36 chromosomal locus disruption. We observed no significant difference in the expression of TAp73 in FL cases with or without chromosome 1p36 disruption (Figure 2A). However,

Np73 expression (Figure 2B) was significantly increased in FL cases with 1p36 abnormalities compared to those without 1p36 abnormality. As the balance between the two opposing p73 isoforms regulates the net cellular response of p73, we evaluated the ratio of Np73:TAp73. The Np73:TAp73 ratio was significantly higher in FL cases with abnormal chromosomal 1p36 than in FL cases with a normal 1p36 (Figure 2C).

To confirm that the observed p73 isoform mRNA expression pattern is reflected at the corresponding TAp73 and Np73 proteins, tissue array IHC analysis was performed. Our data demonstrates a significantly greater expression of Np73 protein in the FL with 1p36 abnormalities ($n = 5$) compared to those without 1p36 abnormality ($n = 5$) (Figures 3A and B). In contrast, there was no significant difference in TAp73 protein expression between FL cases with or without chromosome 1p36 abnormality (Figures 3A and B). Together, these data demonstrate that chromosome 1p36 abnormality leads to higher Np73 expression and Np73:TAp73 ratio in FL.

Increased cellular proliferation in FL cases with 1p36 abnormalities and its association with Np73 expression

Next we analyzed the cellular proliferation in FL cases with and without chromosome 1p36 abnormality using Ki-67 and PCNA immunostaining. Tissue array IHC analysis of Ki-67 expression revealed a significantly higher percentage of Ki-67 positive cells in FL with 1p36 abnormalities ($n = 5$) compared to those without 1p36 abnormality ($n = 5$) (Figure 4A and B). Similarly, IHC analysis for PCNA expression showed a significantly higher percentage of PCNA positive cells in FL with 1p36 abnormalities compared to those cases without 1p36 abnormality (Figures 4A and B). Importantly, a significant positive correlation between Np73 and Ki-67 ($r = 0.689$; and $p = 0.027$) or PCNA ($r = 0.532$; $p = 0.041$) expression was observed, suggesting that imbalance in p73 expression modulates cellular proliferation in FL.

Decreased apoptosis in FL with 1p36 abnormalities and its association with the p73 isoform expression

We examined whether chromosome 1p36 abnormality is associated with cellular apoptosis. Tissue array IHC analysis for cleaved caspase-3 expression showed a significantly lower percentage of cleaved caspase-3 positive cells in FL with 1p36 abnormalities ($n = 5$) as compared to those without 1p36 abnormality ($n = 5$) (Figures 4A and B). We observed a significant negative correlation between the Np73:TAp73 ratio (Np73 IHC score/TAp73 IHC score) and cleaved caspase-3 expression ($r = -0.614$; $p = 0.015$).

Decreased expression TAp73 and its pro-apoptotic targets Bim, Puma, and Noxa in FL compared to FH

To explore the biological significance of p73 in the pathogenesis of FL, the expression of TAp73 and its pro-apoptotic transcriptional targets was compared between FL and its benign counterpart, reactive follicular hyperplasia (FH). Real-time PCR revealed a significantly lower expression of TAp73 in FL ($n = 11$) compared to FH ($n = 5$) (Figure 5). However, there was no significant difference in Np73 expression or the Np73:TAp73 ratio between FL and FH samples. At the protein level, tissue array IHC analysis also revealed significantly lower TAp73 expression in FL as compared to FH (Figures 5B and C) with a significantly higher Np73:TAp73 ratio in FL.

When mRNA expression of the three pro-apoptotic transcriptional targets of p73 (Bim, Puma, and Noxa) was evaluated, the expression of all the three genes was significantly lower in FL ($n = 11$) than FH ($n = 5$); Bim, Puma, and Noxa (Figure 6). Interestingly, we

observed no significant difference in the expression of BIM, PUMA and NOXA in FL cases with and without 1p36 deletion (data not shown), which was similar to TAp73 expression pattern in these cases (Figure 2A). Furthermore, non-parametric correlation analysis of FL samples in the present study demonstrated correlation between TAp73 expression and the expression of Puma ($r = 0.397$, $p = .03$) and Noxa ($r = 0.456$, $p = .01$) indicating that the low expression of TAp73 in FL is reflected in its pro-apoptotic targets.

Discussion

Rearrangements of the chromosomal region 1p36 have been reported to be a common event in a wide variety of tumors including epithelial, neural, and hematological cancers (15). In our previous study of 850 of cases NHL, 1p36 chromosomal rearrangements were found in 12% of the cases (5). In all of these cases, 1p36 rearrangements were associated with other cytogenetic abnormalities and never occurred as the sole abnormality suggesting, a role in disease progression rather than tumor initiation (5). In the present study, we demonstrate that 1p36 disruption deregulates p73 isoform expression by up-regulating Np73 in tumor cells, resulting in decreased apoptosis and increased proliferation and thus explaining the adverse prognostic effect of 1p36 disruption in FL (6).

The prognostic impact of 1p36 abnormalities was demonstrated in a study of 106 cases of FL, wherein 1p36 abnormalities were an independent predictor of inferior survival and a high risk of transformation (6). These data suggested the presence of one or more critical genes in this chromosomal region. There are many genes mapping to the 1p36 region that are relevant to lymphoma biology, including mutations of TNFRSF14 which were found to be important in FL (16). *TP73* is one of the most distally located genes in the 1p36.3 locus and, hence, is vulnerable to disruption during chromosomal rearrangement involving the 1p36 locus. Heterozygous deletions of the *TP73* locus have been reported in NHL (10). The *TP73* gene has also been shown to be abnormally expressed in NHL (17). Furthermore, a recent study found that the most recurrent breakpoint in a series of hematologic malignancies harboring 1p36 rearrangements was in the *TP73* locus (18). However, the effect of 1p36 rearrangement on the expression of p73 isoforms was not evaluated. Also, the biological significance of *TP73* in FL has not been studied. Therefore, we examined whether 1p36 abnormalities modulate the expression of the two major classes of isoforms generated at the *TP73* gene locus (TAp73 and Np73), and investigated whether this could impact p73 function in FL. To determine if 1p36 abnormalities alter the differential p73 isoform expression profile, we compared the mRNA expression patterns of both isoforms in FL cases with or without 1p36 abnormalities. Interestingly, we observed a significant up-regulation in the mRNA and protein expression of Np73 in FL with 1p36 abnormalities compared to those without 1p36 abnormality. In contrast, there was no significant difference in the mRNA or protein expression of TAp73 between FL cases with or without 1p36 abnormalities. These two classes of isoforms have antagonistic effects on cellular proliferation and apoptosis and their balance controls cell fate. Therefore, it has been suggested that the ratio of the two opposing classes of isoforms determines the net cellular response (19;20). Therefore, we also compared the Np73:TAp73 ratios between the two groups, and found that the Np73:TAp73 ratio was significantly higher in FL with abnormal 1p36 compared to those without 1p36 abnormality. These results suggest for the first time

that 1p36 chromosomal abnormalities differentially modulate p73 isoform expression in FL with increased Np73 expression and a higher Np73:TAp73 ratio.

To investigate whether 1p36 abnormalities and the associated differential p73 isoform expression correlate with cellular function, we evaluated cellular proliferation and apoptosis in FL cases with or without 1p36 chromosomal abnormalities by IHC. For evaluation of proliferation, we studied the expression of Ki-67 protein, a nuclear antigen associated with proliferation, with Ki-67-expressing cells reflective of the proportion of actively proliferating lymphoma cells. Ki-67 staining has been correlated with aggressive behavior and a poor prognosis in some NHL subtypes (21). Proliferation was significantly higher in FL cases with 1p36 abnormalities as compared to those without 1p36 abnormality. A relationship between p73 and Ki-67 expression in NHL was reported in a previous study, in which the authors found higher Ki-67 expression in cases with heterozygous deletion of the *TP73* locus (10). In the present study, Ki-67 expression was significantly correlated with Np73 expression. To further strengthen this finding, we also tested the proliferation marker PCNA (22), and observed a similar trend to Ki67 with a higher percentage of PCNA positive cells in FL with 1p36 abnormalities as compared to those without 1p36 abnormality. We also observed a significant correlation between Np73 expression and PCNA.

For assessment of apoptosis, we studied cleaved caspase-3 protein expression by IHC. Cleaved caspase-3 is the active form of a downstream effector caspase in the apoptotic machinery, representing a final common place where multiple apoptotic pathways converge. Immunostaining for cleaved caspase-3 has been found to correlate with better overall survival in diffuse large B-cell lymphoma (23). In our study, the percentage of caspase-3 expressing cells was lower in FL with 1p36 abnormalities compared to those without 1p36 abnormality. In addition, there was a significant negative association between caspase-3 expression and the Np73:TAp73 ratio, thus linking the p73 expression profile with cleaved caspase-3 expression.

In order to further evaluate the relevance of p73 to the pathogenesis of FL, FH samples were used to explore whether the p73 isoform expression profile differs between FL and FH. At both the mRNA and protein level, TAp73 expression was significantly lower in FL compared to FH, suggesting a potential role for TAp73 in the pathogenesis of FL. However, there was no significant difference in the expression of Np73 mRNA or protein between FL and FH. In a study of normal tonsillar B-cells, temporal up-regulation of Np73 isoforms has been suggested at specific B cell developmental stages (24). The expression of Np73 isoforms was observed in activated and germinal center B cells compared to very low levels in memory B cells and follicle mantle cells (24).

Next, to decipher the molecular impact of the low TAp73 expression on net p73 function, a panel of three transcriptional targets downstream of p73, including Bim (bcl-2 interacting mediator of cell death), Puma (p53 upregulated modulator of apoptosis), and Noxa were compared between FL and FH. Bim, Puma, and Noxa belong to the Bcl-2 homology (BH3)-only subgroup of the Bcl-2 family. These three members of the Bcl-2 family are pro-apoptotic, exerting their action through binding to and inhibiting anti-apoptotic members of

the family such as Bcl-2, as well as binding to and activating effector pro-apoptotic members in the family such as Bak and Bax (25). Bim has also been shown to be a target of p73 in regulating mitotic cell death (26) and activation-induced T-cell death (27). Interestingly in the former study that investigated the role of p73 in regulating mitotic cell death, p73 but not p53 knock out diminished the expression of Bim and decreased taxol-induced cell death indicating that p73 but not p53 is the major regulator of Bim. Puma is reported to be a transcriptional target of p73, playing important role in p73-induced apoptosis (28;29). Puma was reported to be a major regulator of memory B-cell survival (30). Also, Noxa is induced by p73 upon exposure to chemotherapy (31). Puma and, to a lesser extent, Noxa can suppress Myc-induced lymphomagenesis in animal models (32). In the present study, Bim, Puma, and Noxa were all significantly down-regulated in FL as compared to FH samples. Interestingly, we observed no significant difference in the expression of BIM, PUMA and NOXA in FL cases with and without 1p36 deletion (data not shown), which was similar to TAp73 expression pattern in these cases (Figure 2A). Furthermore, non-parametric correlation analysis of FL samples in the present study demonstrated correlation between TAp73 expression and the expression of Puma ($r = 0.397$, $p = .03$) and Noxa ($r = 0.456$, $p = .01$) indicating that the low expression of TAp73 in FL is reflected in its pro-apoptotic targets.

In conclusion, we report that dysregulated p73 isoform expression has functional effects that may, at least in part, explain the aggressive behavior and poor prognosis reported in FL cases with 1p36 chromosomal abnormalities (6). Considering the diverse minimum deleted regions encompassing different candidate genes on 1p36 and the complex karyotypes of many FL cases, it is possible that more than one of these genes orchestrate the molecular consequences of 1p36 chromosomal abnormalities in FL. However, our findings emphasize the role of 1p36 chromosomal rearrangement in the differential regulation of p73 isoform expression, and suggest an important role for p73 in the biology of FL that warrants further investigation. Further analysis of the role of p73 may lead to its use as a prognostic marker and therapeutic target in FL.

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Figure 1. Characterization of the 1p36 status in FL

Representative images of confirmatory FISH studies to determine the presence or absence of 1p36 abnormalities in FL using locus-specific probes for 1p36 (LSI 1p36 = red) and the control region 1q25 (LSI 1q25 = green). A) Confirmation of 1p36 locus deletion illustrated by absence of one red signal (LSI 1p36) and the presence of two green signals (LSI 1q25) in a FL with 1p36 deletion. B) Retention of both copies of 1p36 and 1q25 in a FL without 1p36 abnormality.

Figure 2. 1p36 chromosomal abnormalities modulate the p73 isoform mRNA expression profile
 Quantitative real-time PCR analysis comparing the expression of A) TAp73, B) Δ Np73, and C) the ratio of Δ Np73:TAp73 between FL with ($n = 10$) and without ($n = 10$) 1p36 abnormalities. The cycle threshold (ct) values were normalized to those of the housekeeping gene β -actin. Breaks in the Y-axis as well as in the bars were used to accommodate for large values in Figure 2.B and C figures.

Figure 3. Differential p73 isoform protein expression in FL with and without 1p36 abnormalities
 A) Representative IHC stains (X 200) showing increased expression of Δ Np73 protein in a FL with abnormal 1p36 compared to a FL without 1p36 abnormality. B) Quantitative analysis of IHC scores for Δ Np73 and TAp73 expression in FL biopsies with abnormal 1p36 ($n = 5$) compared to those with normal 1p36 ($n = 5$).

Figure 4. Proliferation and apoptosis in FL with and without 1p36 abnormalities

A) Representative IHC stains (X 200) showing increased expression of Ki-67 and PCNA and decreased expression of cleaved caspase-3 in a FL with abnormal 1p36 compared to a FL without 1p36 abnormality. B) Quantitative analysis comparing the percentages of Ki-67, PCNA, and cleaved caspase-3 positive cells in FL with abnormal 1p36 ($n = 5$) compared to FL without 1p36 abnormality ($n = 5$).

Figure 5. TAp73 expression in FL and FH

A) Quantitative real-time PCR analysis comparing the expression of TAp73 in FL ($n = 10$) and FH ($n = 5$). The cycle threshold (ct) values were normalized to those of the housekeeping gene β -actin. B) Quantitative analysis of IHC scores for TAp73 expression in FL ($n = 5$) and FH ($n = 5$). C) Representative IHC stains (X 200) of a FL showing decreased expression of TAp73 protein compared to FH.

Figure 6. mRNA expression of p73-dependent transcriptional pro-apoptotic target genes
Quantitative real-time PCR analysis comparing the expression of Bim, Puma, and Noxa in FL ($n = 10$) and FH ($n = 5$). The cycle threshold (ct) values were normalized to those of the housekeeping gene β -actin.