Molecular Immunology 48 (2011) 408–414

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Epigenetic mechanisms regulate *ΔNP73* promoter function in human tonsil B cells

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ARTICLE INFO

Article history:
Received 1 August 2010
Received in revised form 23 August 2010
Accepted 1 September 2010
Available online 3 December 2010

Keywords:
B-cells
TP73
Epigenetics
DNA methylation

ABSTRACT

ΔNp73 is an anti-apoptotic product of the *TP73* gene whose function in the immune system has not been extensively studied. We analyzed human tonsil B cell subpopulations physically subdivided into resting or activated fractions and found ΔNp73 gene expression essentially in cells bearing features of activation. Moreover, and accordingly, both these fractions proved to be sensitive to treatment in culture with the polyclonal activator TPA that caused substantial increase in ΔNp73 mRNA and protein expression.

We also analyzed the *TP73* oncogenic-relevant internal promoter 2 (P2) and identified epigenetics as its major regulatory factor since active DNA and histone configurations strictly correlated with ΔNp73 expression upon activation by agents capable of loosening chromatin compaction.

Finally, in line with the known TPA pathway, we found that nuclear proteins could bind a sequence corresponding to a unique AP1 site on promoter 2 selectively in the activated cell fraction.

Our results suggest a ΔNp73 function in B cell immunity, indicate epigenetics as master *TP73* P2 regulator, and point to AP1 site occupancy as playing an putative mechanistic role in this process.

1. Introduction

The *TP73* gene produces many different transcripts and proteins that may exert opposite functions (Melino et al., 2002; Stiewe, 2007). ΔNp73 is one of the N terminus-truncated forms of p73 and results either from alternative splicing of a transcript derived from the upstream P1 promoter (*ΔN′p73*) or from the usage of an internal P2 promoter (*ΔNp73*), located upstream of exon 3′.

ΔNp73 is considered an oncogenic protein and appears to be implicated in a variety of human cancers. Its strong expression in tumors has been repeatedly reported as an independent negative predictor of disease outcome and of response to therapy (Casciano et al., 2002b; Stiewe et al., 2004; Zaika et al., 2002). Given its potential oncogenic nature, ΔNp73 in normal tissues appears to be expressed mainly in restricted conditions (Cabrera-Socorro et al., 2006; Grob et al., 2001; Yang et al., 2000).

ΔNp73 expression can be induced by p53 and by the full length TAp73, but, through a negative feedback loop, it can act either as a dominant-negative regulator of these genes (Grob et al., 2001; Nakagawa et al., 2003; Vossio et al., 2002) or by direct activation of its own target genes.

Genetic and epigenetic alterations of the *TP73* gene have been described in leukemia and lymphoma of different lineages (Bueso-Ramos et al., 2005; Corn et al., 1999; Kawano et al., 1999; Novak et al., 2001; Sato et al., 2010; Siu et al., 2002) and the overexpression of *TP73*, a common feature of B-CLL, might be involved in tumorigenesis by changing the ratio between the oncogenic and anti-oncogenic variants of *TP73* (Leupin et al., 2004; Novak et al., 2001). In non-tumoral lymphocytes, p73 function has already been found essential for antigen-induced death of circulating peripheral T cells after T-cell receptor activation of thymocyte (Lissy et al., 2000). Conversely, the survival of antigen-stimulated T cells requires the inhibition of p73 expression that could result from the activity of N-truncated forms (Wan and DeGregori, 2003). Differently from T-lymphocytes and from leukemia and lymphoma only scanty information is available on the expression of p73 family members in normal B-cells. We thus set out to examine, in normal B-cell subpopulations, the mechanisms regulating the function of the internal P2 promoter from which the anti-apoptotic *ΔNp73* variant is transcribed.

2. Materials and methods

2.1. Isolation, fractionation and cell cultures of tonsil B cell

Mononuclear cells were isolated from tonsils of 5–12-year-old children by Ficoll-Hypaque density gradient centrifugation.

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doi:10.1016/j.molimm.2010.09.001

Table 1
Primers and PCR conditions utilized for qPCR, pyrosequencing and ChIP reactions and PCR conditions.

Primer	Forward	Reverse
qPCR		
P1 (*ΔN′p73*)	GGATTCCAGCATGGACGTCTT	AGAGGCTCCGCAGCTAGTGA
P2 (*ΔNp73*)	GGAGATGGGAAAAGCGAAAAT	GTGGACCGAGCGGGAGAG
Pyrosequencing		
PCR Amplification	ATAGAATTAAGGGAGATGGGAAAA	R-AAAAAATCCTATAAATAAC
Sequencing	TTYGGTTTTTGTTTTATTAGTTG	
ChIP		
AP1	CCAAGACGGCTGAAATACAATGG	AAAGCAGGGGTGAGTCCAAGAG

qPCR conditions: initial denaturation 95 °C – 10 min; 40 cycles; denaturation: 95 °C – 15 s; annealing and extension: 60 °C – 60 s.
PCR amplification conditions for pyrosequencing: initial denaturation: 95 °C – 10 min; 45 cycles; denaturation: 95 °C – 30 s; annealing: 51.5 °C – 30 s; extension: 72 °C – 30 s. The analyzed sequence was: YGGAGTTTTTTTYGTTYGGTTTAYGTTGTYGGGYGGTTAYGAT. R-: biotinylated primer. Y: C/T.
ChIP for AP1: initial denaturation: 95 °C – 10 min; 35 cycles; denaturation: 95 °C – 30 s; annealing: 60 °C – 30 s; extension: 72 °C – 30 s.

Informed consent was signed by the parents of the donors. T lymphocytes were removed from the cell suspensions as rosettes with sheep red blood cells by density gradient centrifugation.

Germinal center (GC), activated (ACT), memory (MEM) and follicular mantel (FM) B cell subpopulations were isolated by FACS cell sorting according to a method first described by Pascual et al. (1994). Briefly, highly purified B cells were incubated with APC-conjugated anti-CD19, PE-Cy5-conjugated anti-CD38, PE-CY7-conjugated anti-CD3 (Becton Dickinson, Buccinasco, Italy) and anti-IgD (Dako Cytomation, Glostrup, Denmark). The CD19+ B cells were gated and subsequently FACS sorted (FacsARIA, Becton Dickinson, Buccinasco, Italy) according to the expression of CD38 and IgD (Cutrona et al., 2006).

For functional studies, tonsil B cells were density-fractionated on discontinuous Percoll gradients. The 60% Percoll fraction contained small resting high density (HD) B cells while the 50% Percoll fraction contained large activated, low density (LD) B cells as previously determined (Cutrona et al., 2006; Dono et al., 1996, 2007). To test ΔNp73 expression following in vitro activation, HD cells and LD cells were seeded at 2×10^6 cells/well in 24-well plates in the presence of phorbol myristate acetate (TPA, 10 ng/mL, Sigma–Aldrich, Milan, Italy) or 1 μM Tricostatin A (TSA, Sigma–Aldrich, Milan, Italy) for the time indicated in the text.

To monitor the effect of demethylating agents on *ΔNp73* expression, Raji cells were treated with 5 μM 5-Azacytidine for 96 h. The effect of the treatment was verified by qPCR and by pyrosequencing as described in Sections 2.3 and 2.4.

2.2. Western blot analysis

Whole cell extracts from the different cell types were obtained after exposure to lysis buffer (9 M urea, 50 mM Tris/HCl pH 7.5). Proteins concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Segrate, Italy). A constant amount of proteins (40 μg) was separated by SDS-PAGE on 10% acrylamide gels and transferred to polyvinylidene fluoride (PVDF) Hybond–P membranes (GE Healthcare, Milano, Italy). The membranes were then blocked and exposed to the anti ΔNp73 monoclonal antibody IMG313 (Imgenex, S. Diego, CA).

Control antibody was against β-actin (Sigma–Aldrich, Milan, Italy). Secondary antibody was an anti-mouse Ig antibody conjugated with peroxidase (HRP, Santa Cruz Biotechnology Inc., Santa Cruz, CA). Peroxidase activity was revealed by chemiluminescence (GE Healthcare, Milano, Italy).

2.3. qPCR analysis

Total RNA and DNA were extracted using AllPrep DNA/RNA Kit (Qiagen, Milano, Italy). Fifty nanogram of Total RNA were used to prepare amplified cDNA using WT-OVATION™ RNA Amplification System (NuGEN Technologies, San Carlos, CA). The sequence of the primers and PCR conditions are reported in Table 1. GAPDH was utilized for normalization. (PrimerDesign Ltd, Southampton, UK). Two independent quantitative PCR reactions were performed for each sample. Each sample was analyzed in triplicate and only PCR reactions with an intraassay S.D. for Cq variance <0.2 were considered.

2.4. Methylation analysis of TP73 promoter 2

Quantitative methylation analysis by pyrosequencing was performed with a SPQ 96MA apparatus (Biotage, Uppsala, Sweden) (Banelli et al., 2010; Tost and Gut, 2007). Bisulfite-modified DNA was amplified with primers designed with the Assay Design Software (Biotage, Uppsala, Sweden). The primer sequences and the PCR conditions are reported in Table 1. Universally methylated and unmethylated control DNA was obtained from Chemicon (Temecula, CA, USA). Two independent pyrosequencing reactions were performed for each sample.

2.5. Chromatin immunoprecipitation (ChIP)

ChIP for methylated K4 and K27 Histone 3 was performed with the Epiquik strip microplate kits (Epigentek, NY) according to manufacturer's instructions. ChIP for AP1 complexes was performed with a rabbit polyclonal antibody directed against c-Jun (H-79*) (Santa Cruz Biotechnology, Santa Cruz, CA). Primers for AP1 binding site are reported in Table 1. Relative PCR band intensity was analyzed by densitometry and expressed as percentage of input signal. Negative control with a normal mouse or rabbit IgG (as appropriate) and a PCR control using whole genome as a template were always included.

2.6. Electrophoresis mobility shift assay (EMSA) analysis

Nuclear extracts were prepared according to published procedures (Andrews and Faller, 1991). Twenty microgram of extract were added to 10 pmol of P^{32} labelled probe corresponding to a minus strand AP1 binding site (AF443116.1-sequence TGAGGTGACTGCCACTCCCTG; Genomatix MatInspector 3.3) identified at position −701/−721 from nucleotide 1 of Exon 3′, the starting exon of *ΔNp73* (Casciano et al., 2002a). After 20′ incubation at room temperature, complexes were separated on a 5% polyacrylamide gel, dried and developed overnight.

2.7. Promoter assay

The *ΔNp73* promoter (Casciano et al., 2002a) comprising nucleotides −952/+50, corresponding to nucleotides 3084916–3085917 of the Chromosome 1 sequence, hg build 18 was cloned into the pGL3-basic luciferase reporter vector