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Impact of DNA methyltransferases on the epigenetic regulation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor expression in malignant melanoma

Mario Venza [a,1], Maria Visalli [b,1], Teresa Catalano [b], Cinzia Fortunato [b], Rosaria Oteri [b], Diana Teti [b,*], Isabella Venza [a]

[a] Department of Experimental Specialized Medical and Surgical and Odontostomatology Sciences, University of Messina, Messina, Italy
[b] Department of Clinical and Experimental Medicine, University of Messina, Messina, Italy

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ABSTRACT

Aberrant promoter methylation and resultant silencing of TRAIL decoy receptors were reported in a variety of cancers, but to date little is known about the relevance of this epigenetic modification in melanoma. In this study, we examined the methylation and the expression status of TRAIL receptor genes in cutaneous and uveal melanoma cell lines and specimens and their interaction with DNA methyltransferases (DNMTs) DNMT1, DNMT3a, and DNMT3b. *DR4* and *DR5* methylation was not frequent in cutaneous melanoma but on the contrary it was very frequent in uveal melanoma. No correlation between methylation status of *DR4* and *DR5* and gene expression was found. *DcR1* and *DcR2* were hypermethylated with very high frequency in both cutaneous and uveal melanoma. The concordance between methylation and loss of gene expression ranged from 91% to 97%. Here we showed that DNMT1 was crucial for *DcR2* hypermethylation and that DNMT1 and DNMT3a coregulate the methylation status of *DcR1*. Our work also revealed the critical relevance of *DcR1* and *DcR2* expression in cell growth and apoptosis either in cutaneous or uveal melanoma. In conclusion, the results presented here claim for a relevant impact of aberrant methylation of decoy receptors in melanoma and allow to understand how the silencing of *DcR1* and *DcR2* is related to melanomagenesis.

1. Introduction

In recent years it has been shown that epigenetic alterations are causally related to melanomagenesis in addition to genetic mutations [1]. The regional hypermethylation of CpG islands within the promoter region of tumor suppressor genes (TSGs) associated with transcriptional downregulation appears to be the epigenetic mechanism mostly studied in the pathogenesis of melanoma [2,3]. Among TSGs tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is particularly important, because it can induce apoptosis in a wide range of cultured malignant cells, but not in normal tissues [4]. For this reason the potential of TRAIL as an anticancer agent has been investigated in animal models [5]. Four key human receptors for TRAIL have been identified, of which TRAIL-R1 (DR4/TNFRSF10A) and TRAIL-R2 (DR5/TRICK2/TNFRSF10B) contain an intracellular death domain that can activate classic death signaling [6,7] and TRAIL-R3 (TRID/DcR1/TNFRSF10C) and TRAIL-R4 (DcR2/TNFRSF10D), also named decoy receptors, do not transduce apoptotic signals, as the former lacks a cytoplasmic domain [6] and the latter contains a truncated cytoplasmic death domain [8]. Indeed, the proteins encoded by TRAIL decoy receptors have been shown to play an inhibitory role in TRAIL-induced apoptosis [9]. Despite the relevant role of TRAIL in cancer, few data concerning epigenetic alterations of genes encoding for TRAIL receptors in melanoma are available. A study showed that promoter hypermethylation of *DR4* reduced the sensitization of melanoma to the IFN-α2b- and IFN-β-induced apoptosis [10] and another one reported that *DcR2* is a gene frequently silenced by methylation in melanoma [11]. On the contrary, *DcR1* and *DR5* have never been assessed for their methylation status in this cancer. The methylation machinery consists of three known catalytically active DNA methyltransferases (DNMTs), DNMT1, DNMT3a, and DNMT3b. During DNA replication, DNMT1 can recognize the normally methylated CpG sites in the parent strand and catalyze cytosine methylation in the corresponding CpG site of the daughter strand [12]. DNMT3a and DNMT3b are responsible for *de novo* methylation [13]. An increased DNMT expression was associated with cancer progression [14,15], but at the moment no data on this

* Corresponding author. Address: Dipartimento di Medicina Clinica e Sperimentale, Azienda Policlinico Universitario, Torre Biologica – 4° piano, via Consolare Valeria, 1, 98125 Messina, Italy. Fax: +39 90 2213341.
E-mail address: dteti@unime.it (D. Teti).
[1] These authors contributed equally to this work.

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regard are available for melanoma. To obtain an integrated information on TRAIL receptor methylation-induced silencing and the involvement of DNMTs in melanomagenesis, we have carried out studies on formalin-fixed paraffin-embedded (FFPE) sections and cell lines of cutaneous and uveal melanoma.

2. Materials and methods

2.1. Tissue specimens

Sixty FFPE tissue sections of cutaneous melanoma, 6 of uveal melanoma and 48 normal skin specimens were collected from the Department of Human Pathology, University of Messina, Italy. Patient data are shown in Table 1. The investigation adhered to the Declaration of Helsinki and was approved by the Ethics Committee of the University Hospital of Messina. An informed consent was given by the patients.

2.2. Cell cultures

The cutaneous melanoma cell line GR-M (ECACC, European Collection of Cell Cultures, Salisbury, UK), and the uveal melanoma cell lines OCM-1 (provided by J. Mellon, Department of Ophthalmology, UT Southwestern Medical Center, Dallas, TX), OCM-3, 92.1, and Omm2.5 (provided by Martine J. Jager, Leiden University Medical Center, Leiden, The Netherlands) were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 1% penicillin/streptomycin, and 10% FBS. G361 cutaneous melanoma cells (ECACC) were grown in McCoy's 5a medium modified with 10% FBS, 2 mM L-glutamine, and 1% penicillin/streptomycin.

2.3. Treatment with the DNA demethylating agent 5-aza-dC

Cells were treated with 5-Aza-dC (Sigma Chemical Co.) by addition of fresh medium containing 5-Aza-dC (10 μmol/L) every day for three consecutive days.

2.4. Total RNA and DNA extraction

Total RNA and DNA extraction was performed by RecoverAll Total Nucleic Acid Isolation kit (Ambion Inc.) from FFPE samples, and by TRIzol Reagent (Invitrogen) from cells.

2.5. Reverse transcription and quantitative real-time PCR

Total RNA was reverse-transcribed with IMProm-II™ reverse transcriptase kit (Promega). Quantitative real-time PCR was performed by ABI Prism 7500 real-time PCR System (Applied Biosystems, Milan, Italy). Primers and probes are listed in Table 2. The mRNA levels of specific genes were normalized to endogenous β-actin (Applied Biosystems).

2.6. Bisulfite sequencing and methylation-specific PCR (MSP)

Bisulfite-modified DNA obtained by Epitect Bisulfite kit (Qiagen) was amplified using primers specific for either methylated or unmethylated DNA sequences. PCR products were separated by 2% agarose gel containing ethidium bromide. Primer sequences are reported in Table 3. Detailed methylation status was confirmed by bisulfite sequencing (CEQ 2000 DNA Analysis System, Beckman Coulter Inc.).

2.7. Western blot analysis

Total cell extracts (50 μg) were resolved by SDS–PAGE and blotted onto nitrocellulose membranes with anti-DNMT1, anti-DNMT3a, and anti-DNMT3b antibodies (Santa Cruz Biotechnology).

2.8. Plasmids

The full-length human *DcR1* cDNA (NCBI reference sequence: NM_003841.3) was amplified by PCR with the following primers: forward, 5′-ATGGCCCGGATCCCCAAGA-3′, reverse 5′-TCAAACAAA CACAATCAGAAGCAC-3′. For *DcR2* (GenBank: AF029761.1), the forward primer sequence was 5′-ATGGGACTTTGGGGACAAAGCG TCC-3′, and the reverse primer was 5′-TCACAGGCAGGACGTAGCA-GAGCC-3′. The resulting 780-bp (DcR1) and 1161-bp (DcR2) PCR products were cloned into the pcDNA3.1 vector (pcDNA™3.1 Directional TOPO® Expression Kit, Invitrogen). The constructs were verified by DNA sequencing (CEQ 2000 DNA Analysis System).

2.9. Transient transfections

Cells were transiently transfected with siDNTM1, siDNMT3a, siDNMT3b, and control siRNA (Qiagen), or with DcR1 and/or DcR2 expression vectors using METAFECTENE® PRO transfection reagent (Biontex Laboratories GmbH, Germany).

2.10. Chromatin immunoprecipitation (ChIP)

ChIP enzymatic assay (Active Motif) was carried out and the sheared chromatin samples were used for immunoprecipitation with anti-DNMT1, anti-DNMT3a, anti-DNMT3b antibodies (Santa Cruz Biotechnology) as previously reported [16]. The primers used in the PCR reaction were: forward, 5′-GGTTTAAGAAGAGGAGAGA-CAGG-3′ and reverse, 5′-TCACTTCCAAGCACTCAGAAAAG-3′ for DcR1 (GenBank: AF524869.1); forward, 5′-GGCAGTGTAGCTGCGA-GAACCTT-3′ and reverse, 5′-TGAGAAGGGAGGAGGGTGGATC-3′ for DcR2 (NCBI reference sequence: NG_032579.1). PCR products were separated by 2% agarose gel containing ethidium bromide.

Table 1
Patient characteristics.

Cutaneous melanoma ($n = 60$)		Uveal melanoma ($n = 6$)	
Males	37 (61.7%)	Males	4 (66.7%)
Females	23 (38.3)	Females	2 (33.3%)
Mean age at diagnosis (±SD)	63.83 ± 12.02	Mean age at diagnosis (±SD)	62.45 ± 8.09
Tumor location		*Tumor location*	
Trunk	38 (63.3%)	Choroid	4 (66.7%)
Limbs	13 (21.7%)	Ciliary body	2 (33.3%)
Head	9 (15%)		

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