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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

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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

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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%)		

Table 2
Unmethylated (U) and methylated (M) gene-specific primers, PCR product sizes, and annealing temperatures used for methylation-specific PCR.

Gene methylation	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product size (bp)	Annealing temperature (°C)	Reference
DR4-U	GTAGTGATTTTGAATTTTGGGAGTGTAGT	CTCATAATTCAATCCTCCCCACAA	103	58	18
DR4-M	TTCGAATTTCGGGAGCGTAGC	GTAATTCAATCCTCCCCGCGA	92	56	18
DR5-U	GGTAGTGAAAGTATAGTTGTGTTGT*	ACCCTTAAAATAAATCAAACATCATC*	125	53	–
DR5-M	GAGGTAGTGAAAGTATAGTCGCGTC*	CCCTTAAAATAAATCGAACATCGT*	126	59	–
DcR1-U	TTTTAAGATTTTAAAGTTTGTTGTTGT*	AAACCAAACATCCCTACCATA*	141	51	–
DcR1-M	TTAAGATTTTAAAGTTCGTCGTCGT*	AAAACCAAACATCCCTACCGTA*	140	53	–
DcR2-U	TTGGGGATAAAGTGTTTTGATT	TCACCAACAACAAAACCACA	146	50	18
DcR2-M	GGGATAAAGCGTTTCGATC	CACCGACAACAAAACCGCG*	142	52	18

* Specifically designed for this study.

Table 3
Primers, probe sequences of the genes analyzed by quantitative real time-PCR, and size of amplicons generated.

Gene	Forward primer[a]	Reverse primer[a]	Probe[b]	Product size (bp)
DR4	AGTGCATGGACAGGGTGTGTT	CGTTGCTCAGAATCTCGTTGTG	TTGGGTCTCCTACGAGGG	82
DR5	CAAGACCCTTGTGCTCGTTGT	TTGGGTGATCAGAGCAGACTCA	CCGCGGTCCTGCTGTTGGTCTC	70
DcR1	GGCGTCGGGAACCATACC	ACGATGACGACGACGAACTTT	TGGCCCGGATCCCCAAGACC	62
DcR2	TTGATTTTTGGCGCTTTCG	CGGTCGGGACGCTTTGT	CCACCCTCCTCCCTTCTCATGGGA	70
DNMT1	TGTGAGCAACATAACCAGGTTGA	CAGTGCCGAGGCTCCATT	CGGTGCGAGACACG	103
DNMT3a	CCCGGCTCCAGATGTTCTT	CTGGGACAGGTGGGTAAACCT	ACCAGGAATTTGACCCTC	75
DNMT3b	CATGAAGGTTGGCGACAAGA	TGGCATCAATCATCACTGGATT	CATCTCACGGTTCCTG	68

Sequences for the primers and probes are shown in 5′–3′ orientation.
[a] Designed with Primer Express Software V3.0 (Applied Biosystem).
[b] Probes contain a FAM fluorescent as a reporter at the 5′ end and a MGBNFQ as a quencher at the 3′ end.

2.11. Cell proliferation assay

Cell proliferation was measured using the MTT Assay Kit (Cayman Chemical Company, Michigan, USA).

2.12. Apoptosis assay

Apoptosis was quantified using the Biocolor APOPercentage™ assay (Biocolor Ltd., Newtownabbey, UK).

2.13. Statistical analysis

Comparisons between groups were made with Student's *t* test. The Pearson's correlation test was employed to analyze the correlation between the methylation status of *DcR1* and *DcR2* and the expression level of DNMTs. Differences were considered significant when the *p* value was <0.05.

3. Results

3.1. Methylation and expression levels of DR4, DR5, DcR1, and DcR2 in cutaneous and uveal melanoma

We determined the promoter methylation status of *DR4*, *DR5*, *DcR1*, and *DcR2* in two human cutaneous melanoma cell lines (GR-M and G361), four uveal melanoma cell lines (OCM-1, OCM-3, 92.1, and Omm2.5), and a normal human epidermal melanocyte (NHEM) cell line. As shown in Fig 1A, *DR4* promoter was methylated in uveal, but not in cutaneous, melanoma cells; *DR5* promoter resulted to be methylated exclusively in 3 out of 4 uveal melanoma cells; *DcR1* promoter was methylated in all the cutaneous and uveal melanoma cell lines examined; *DcR2* promoter was methylated in all uveal melanoma cells and in only one cutaneous melanoma line. No promoter methylation was detected for any of the target genes in NHEM cells. We then examined whether hypermethylation would silence or decrease the gene expression and the protein synthesis. As shown in Fig. 1B, *DR4* and *DR5* methylation was not always correlated to a decrease in gene expression. In contrast, aberrant promoter methylation of *DcR1* and *DcR2* resulted throughout in loss of mRNA expression. This reflected the response at the protein level, i.e., that there was a one-to-one correspondence of protein to mRNA (Fig. 1C). Treatment with 5-aza-dC led to demethylation of all promoters (Fig. 1A) and reactivation of *DcR1* and *DcR2* genes (Fig. 1B) and protein synthesis (Fig. 1C). It had no effect on *DR4* and *DR5* gene expression.

We further analyzed the methylation pattern of *DR4*, *DR5*, *DcR1* and *DcR2* in FFPE sections from 60 cutaneous and 6 uveal melanoma patients, and in 48 normal skin specimens. As shown in Fig. 2A, we found that *DR4* was methylated in 11 out of 60 (18.3%) cutaneous melanomas, 6 out of 6 (100%) uveal melanomas, and 7 out of 48 (14.6%) normal skin specimens; *DR5* was methylated in 4 out of 60 cutaneous melanomas (6.6%), 5 out of 6 uveal melanomas (83.3%), and 2 out of 48 (4.2%) normal skin specimens; *DcR1* was methylated in 48 out of 60 cutaneous melanomas (80%), 4 out of 6 uveal melanomas (66.6%), and 6 out of 48 (12.5%) normal skin specimens; *DcR2* was methylated in 49 out of 60 cutaneous melanomas (81.7%), 5 out of 6 uveal melanomas (83.3%), and 5 out of 48 (10.4%) normal skin specimens. The concordance between aberrant methylation and loss of gene expression was 20.8% for *DR4*, 18.2% for *DR5*, 91.4% for *DcR1* and 96.6% for *DcR2* (Fig 2B).

These results show that promoter methylation of *DcR1* and *DcR2* is responsible for the decrease or even the total loss of *DcR1* and *DcR2* expression in melanoma. On the contrary, methylation does not appear to be a mechanism for *DR4* and *DR5* gene silencing in this type of cancer.

3.2. Impact of DNMTs on methylation status and expression of decoy receptor genes

In the light of the concordance between the methylation status and the expression of decoy receptors, we subsequently investigated the involvement of DNMTs on these events. As compared with control NHEM line, *DNMT1* was up-regulated in G361 cells

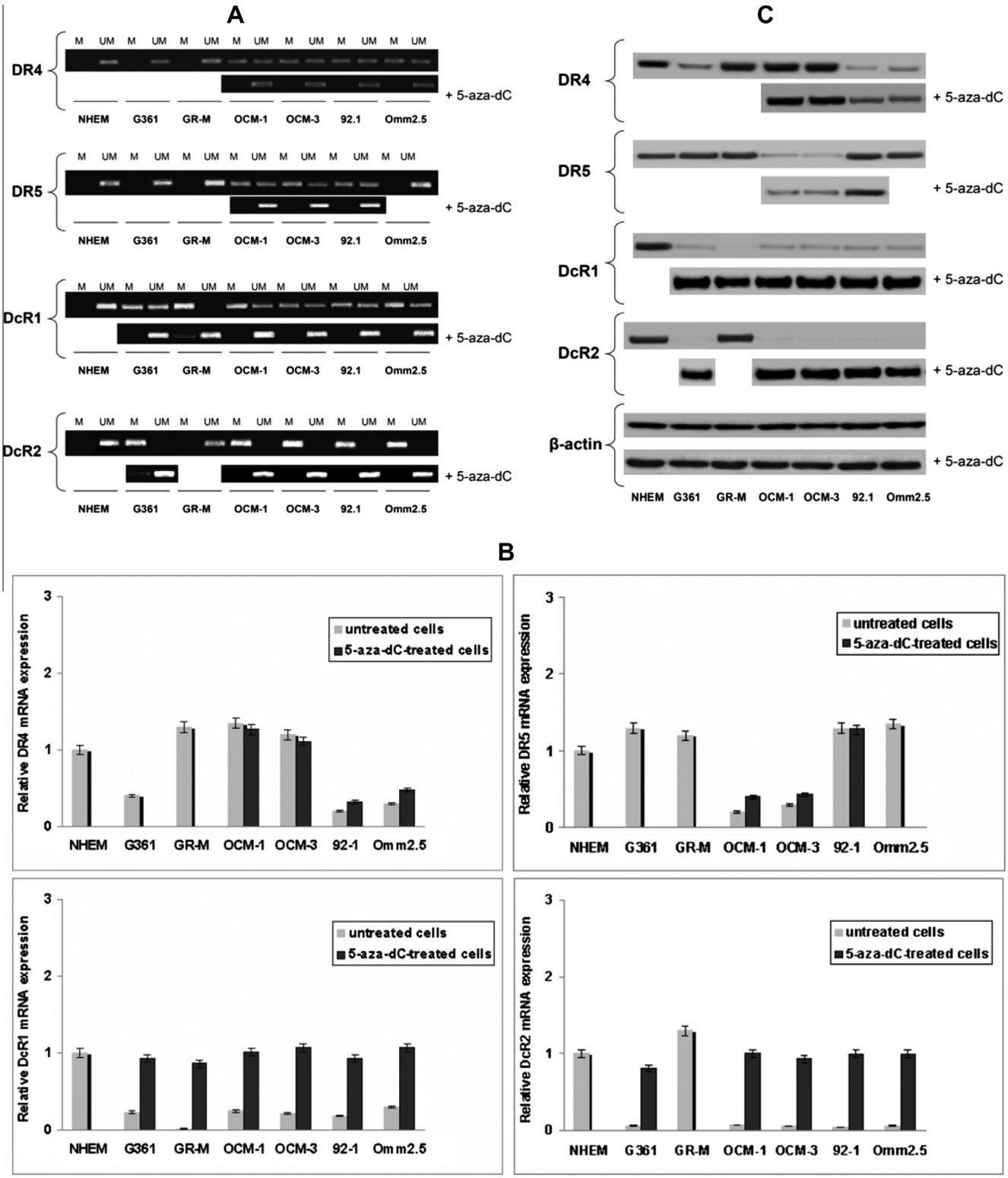

Fig. 1. Methylation analysis and expression levels of *DR4*, *DR5*, *DcR1*, and *DcR2* genes in cutaneous (G361 and GR-M) and uveal (OCM-1, OCM-3, 92.1, and Omm2.5) melanoma cell lines before and after treatment with 5-aza-dC. (A) Promoter methylation status of target genes was determined by methylation-specific PCR (MSP). Extracted DNA was amplified by PCR with primers specific to the unmethylated (UM) or the methylated (M) CpG islands after modification with sodium bisulfite. PCR products were electrophoresed on 2% agarose gel, stained with ethidium bromide, and photographed. The gels shown here represent the typical result from three independent experiments. (B) Total RNA was extracted, reverse-transcribed, and analyzed by quantitative real time-PCR. mRNA levels of target genes were normalized by using the housekeeping gene β-actin as the inner control. Data are depicted as the mean ± SD of three independent experiments. (C) Total protein extracts were subjected to Western blot analysis, as described in Section 2. Blots are representative of three independent experiments.

and in all the uveal melanoma cell lines examined, *DNMT3a* in all the melanoma cell lines, and *DNMT3b* in GR-M cell line and all the uveal melanoma cells. Furthermore, *DNMT1* was overexpressed in 50 of 60 (83.3%) cutaneous melanomas and in 6 of 6 (100%)

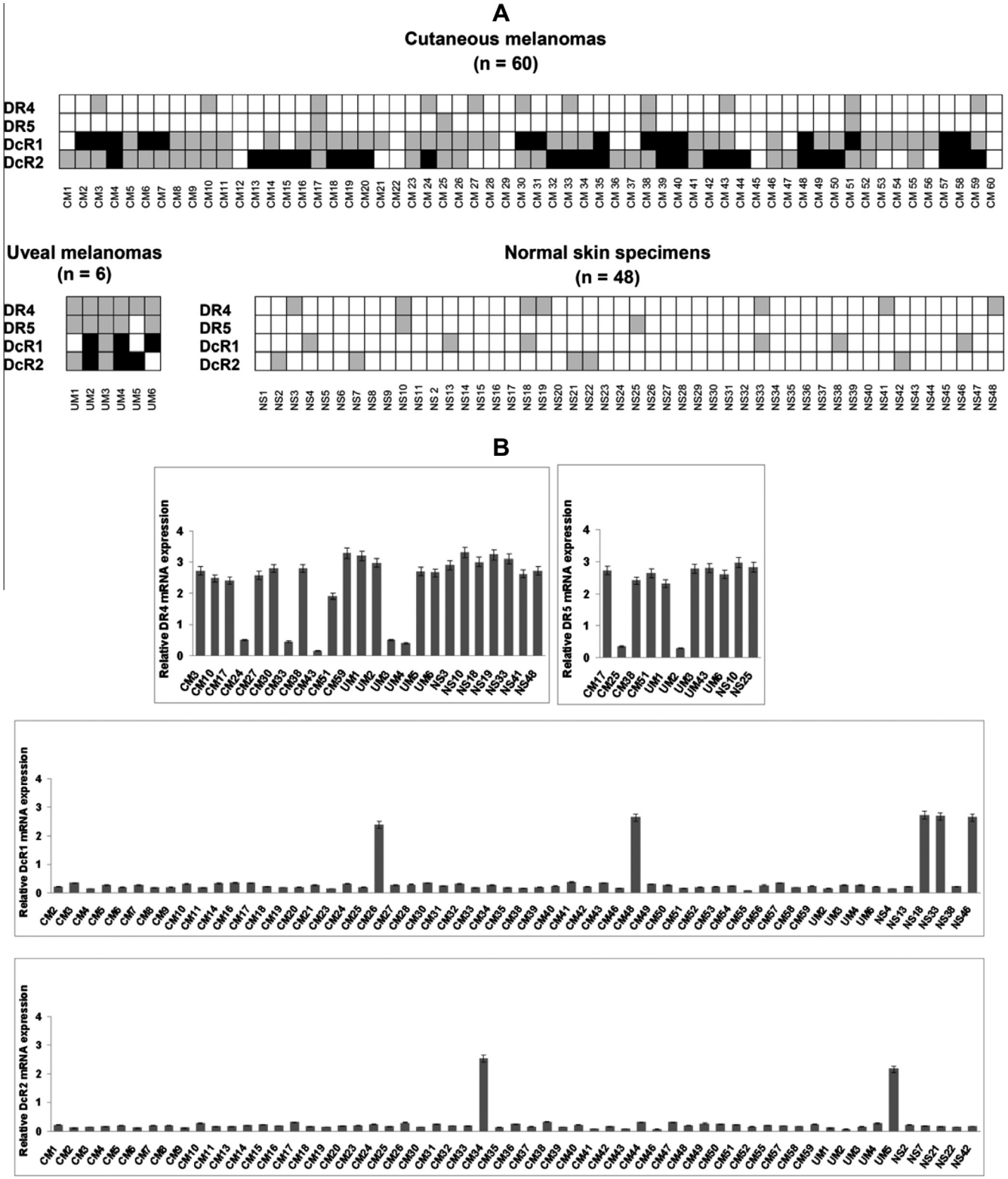

Fig. 2. Methylation status and expression levels of TRAIL receptor genes in cutaneous and uveal melanoma specimens. (A) Detailed methylation pattern of target genes in 60 cutaneous melanomas, 6 uveal melanomas, and 48 normal skin specimens, as determined by methylation specific PCR (MSP). White square, unmethylated CpG regions; grey square, heterozygous methylated CpG regions; black square, homozygous methylated CpG regions. (B) Total RNA was extracted, reverse-transcribed, and analyzed by quantitative real-time PCR. mRNA levels of target genes were normalized by using the housekeeping gene β-actin as the inner control. Data are depicted as the mean ± SD of three independent experiments.

uveal melanoma, *DNMT3a* in 47 of 60 (78.3%) cutaneous melanomas and 5 of 6 (83,3%) uveal melanomas, and *DNMT3b* in 12 of 60 (20%) cutaneous melanomas and 1 of 6 (16.7%) uveal melanomas, as compared to normal skin specimens (see Supplemental Fig. 1). Pearson's correlation test revealed that the methylation status of *DcR1* is positively correlated with the expression levels of

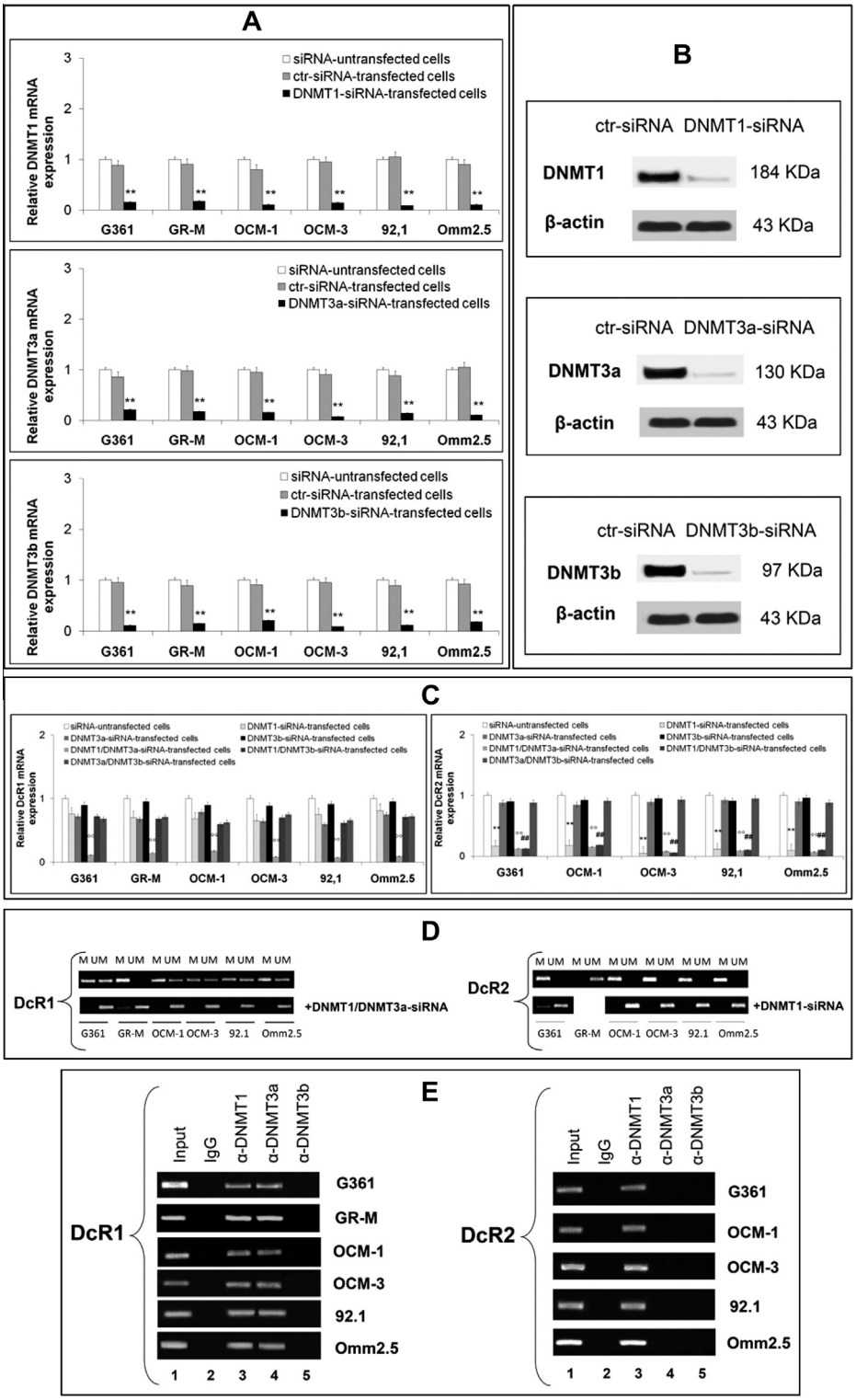

Fig. 3. Impact of DNA methyltransferases (DNMTs) on the methylation status of decoy receptor genes in cutaneous and uveal melanoma cell lines. (A) Cells were transfected with double-stranded DNMT1, DNMT3a and DNMT3b siRNAs and a siRNA control, as described in Methods section. Total RNA was harvested 24 h after transfection and subjected to quantitative real-time PCR. The relative levels of DNMT1, DNMT3a and DNMT3b mRNAs were normalized to β-actin. All data are expressed as mean ± SD from three individual experiments. Significant $^{**}p < 0.01$, siRNA-transfected cells versus untransfected cells. (B) Cellular proteins from siRNA-treated cells were separated by SDS–PAGE for immunoblot analysis with DNMT1, DNMT3a, and DNMT3B antibody. Blots are representative of three independent experiments. As no significant difference was observed in DNMT expression between the cell lines, only data obtained from G361 cells are presented. (C) Cells were transfected with double-stranded DNMT1, DNMT3a and DNMT3b siRNAs, or a combination of them, as described in Methods section. Total RNA was harvested 24 h after transfection and subjected to quantitative real-time PCR. The relative levels of DcR1 and DcR2 mRNAs were normalized to β-actin. All data are expressed as mean ± SD from three individual experiments. Significant $^{**}p < 0.01$, DNMT1-siRNA-transfected cells versus untransfected cells; $^{\circ\circ}p < 0.01$, DNMT1/DNMT3a-siRNA-transfected cells versus untransfected cells; $^{\#\#}p < 0.01$, DNMT1/DNMT3b-siRNA-transfected cells versus untransfected cells, as calculated by Student's *t* test. (D) Extracted DNA was treated with bisulfite and then subjected to methylation-specific PCR (MSP) using the methylated (M) and unmethylated (UM) specific primer sets. Representative results of three different experiments are depicted. (E) DcR1 and DcR2 promoter association with DNMTs was determined by chromatin immunoprecipitation (ChIP) using antibodies directed against DNMT1 (*lane 3*), DNMT3a (*lane 4*), and DNMT3b (*lane 5*). *Lane 1* contains input chromatin. Nonspecific IgG antibody (*lane 2*) was used as a negative control. The gels shown here are representative of three independent experiments.

DNMT1 ($r = 0.706$, $P = 0.0009$) and *DNMT3a* ($r = 0.634$, $P = 0.0087$), and that a strong correlation exists between *DcR2* hypermethylation and *DNMT1* up-regulation ($r = 0.825$, $P < 0.0005$). Conversely, *DcR1* and *DcR2* methylation did not correlate with the expression levels of *DNMT3b*.

We next determined whether siRNA targeted knockdown of DNMTs led to DNA demethylation and re-expression of *DcR1* and *DcR2*. Fig. 3A displays a qreal-time PCR analysis in which siDNMT1, siDNMT3A, and siDNMT3B were used to transfect melanoma cells. A non-functional siRNA was used as a control. We observed a marked decrease in the mRNA levels of DNMT1, DNMT3a and DNMT3b after transfecting cells with specific DNMT siRNAs. Consistent with these results, immunoblot analysis showed that protein levels of DNMT1, DNMT3A and DNMT3B were reduced by the siRNA treatment (Fig. 3B). Combined silencing of DNMT1 and DNMT3a enhanced *DcR1* gene expression, whereas single knockdown of DNMT1 up-regulated *DcR2* (Fig. 3C). In contrast, DNMT3b siRNA treatment did not alter the expression profile of *DcR1* and *DcR2* genes. We also found that double silencing of DNMT1 and DNMT3a was necessary to abolish the methylation of *DcR1* while DNMT1 invalidation alone was sufficient to demethylate *DcR2* (Fig. 3D). To ultimately establish the role DNMTs played on the methylation status of the decoy receptor genes, we examined DNMT occupancy at the *DcR1* and *DcR2* promoters. As illustrated in Fig. 3E, ChIP experiments revealed that DNMT1 was enriched at the methylated *DcR1* and *DcR2* regions, whereas DNMT3a occupied the methylated region of *DcR1* shared by DNMT1. In contrast, no association of DNMT3b above background was observed at either loci. The cross examination of these results indicate that the methylation machinery of *DcR1* gene requires DNMT1/DNMT3a cooperation and that DNMT1 governs the methylation of *DcR2*.

3.3. The tumorigenic potential of melanoma cells is affected by DcR1/DcR2 overexpression

After that we examined the impact of *DcR1*/*DcR2* overexpression on proliferation rate and apoptosis induction in melanoma cells. Overexpression of DcR1 or DcR2 alone led to a significant reduction in the growth rate (Fig. 4A) and a marked increase in the apoptotic response (Fig. 4B). Notably, even greater effects were observed when the *DcR1*/*DcR2* double overexpression was induced. These results indicate that the enforced *DcR1* and/or *DcR2* expression inhibits proliferation and facilitates programmed cell death in melanoma cells, thus diminishing their malignant potential.

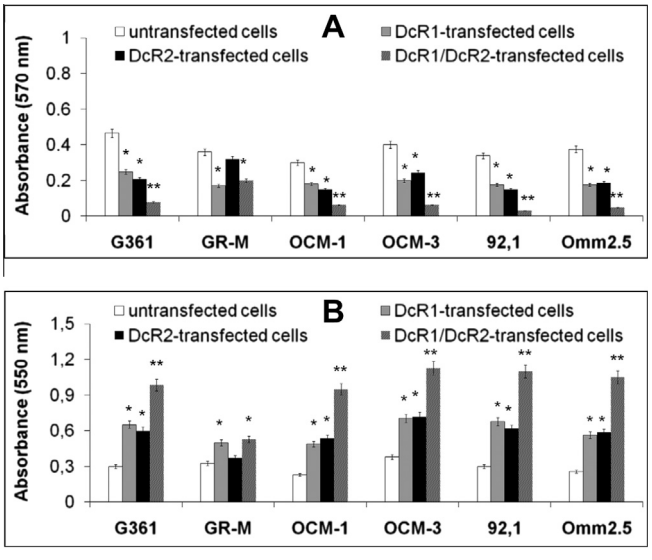

Fig. 4. Effects of ectopic expression of *DcR1* and *DcR2* on cell proliferation and apoptosis. Cells were transiently transfected with *DcR1* and/or *DcR2* expression plasmids. (A) Cell proliferation was assessed by MTT assay. (B) Apoptosis rate was detected by Biocolor APOPercentage™ assay. Results are expressed as mean ± SEM from three separate experiments. Significant $^{*}p < 0.05$ and $^{**}p < 0.01$ versus untransfected cells, as calculated by Student's *t* test.

4. Discussion

Evidence from literature indicate that the DNA methylation-mediated down-regulation of genes involved in apoptosis could be a significant mechanism through which tumor cells avoid apoptosis [17]. Here we showed that TRAIL receptor genes are differently methylated and expressed in cutaneous and uveal melanoma. *DR4* and *DR5* had a low frequency of methylation in cutaneous melanoma and high frequency of methylation in uveal melanoma, but nonetheless the promoter methylation status did not affect gene expression profile (Fig. 1). These data allow to consider that other mechanisms beside methylation operate in cutaneous and uveal melanoma to mediate *DR4* and *DR5* down-regulation. Moreover, it is worth to note that *DR4* and *DR5* resulted in our samples only heterozygous methylated, a condition previously shown by others to be not influent on *DR4* and *DR5* expression [18]. Experiments reported here strongly suggest a significant role of the epigenetic alterations by DNA methylation in the expression of decoy receptors and melanoma behavior. First of all, promoter hypermethylation of *DcR1* and *DcR2* was found in a very high percentage either in cutaneous or uveal melanoma cell lines and specimens. Secondly, we showed that a high positive concordance rate occurred between promoter methylation and lack of the expression of these decoy receptors. Thirdly, 5-aza-dC treatment not only reverted the methylation status of promoters, but also completely restored gene expression (Figs. 1 and 2). Although aberrant methylation and subsequent down-expression of trail decoy receptor genes are frequent in multiple tumor types [19], as far as we may know only one paper reported *DcR2* as a gene frequently methylated and silenced in melanoma [11] and data about the down-regulation of *DcR1* and *DcR2* in melanoma did not report the underlying mechanisms [4]. The methylation of the two decoy receptor appears to occur independently of the adjacent *DR4* and *DR5* genes, since the former were widely methylated in either cutaneous or uveal melanoma, and the latter were infrequently methylated in cutaneous melanoma and frequently methylated in uveal melanoma. So, we can infer that the methylation of the decoy receptors observed by us is not a secondary effect of *DR4* and *DR5* methylation, as seen in other conditions [19]. Furthermore, we were able to identify the DNMTs specifically involved in *DcR1* and *DcR2* methylation. DNMT1 had a critical role in *DcR2* methylation, whereas *DNMT1* and *DNMT3a* cooperation was required for the methylation-induced silencing of *DcR1* (Fig. 3). Indeed, either the involvement of the single DNMT1 or the interaction between *de novo* and maintenance methyltransferases to methylate a specific gene were already been described for other genes in other types of cancer [20,21]. To our knowledge data presented here about the role of DNMts in the methylation and expression of TRAIL receptors in cutaneous as well as uveal melanoma are the first who have dealt with this topic. Given that *DcR1* and *DcR2* have been presumed to function as oncogenes because of their postulated anti-apoptotic effect, and their expression is thought to be advantageous to tumor cells [19], we attempted to explain the significance of decoy receptor silencing in melanomagenesis. To this end we evaluated the effects of *DcR1* and *DcR2* ectopic expression on apoptosis and proliferation of non-expressing melanoma cell lines. The transfection with *DcR1* and/or *DCR2* expression plasmids increased the apoptotic rate and decreased

the cell growth in respect to untrasfected cells, showing that the methylation and the silencing of these genes observed by us may represent a key point in the melanocyte transformation. These data contribute to the debate about the role and the significance of *DcR1* and *DcR2* expression in cancer. At least as regards the melanoma they support the hypothesis that under the conditions prevalent in the tumor microenvironment *DR4* and *DR5* can activate and decoy receptors repress the NF-κB survival pathway [22]. NF-κB pathway triggered by *DR4* and *DR5* is believed to play an anti-apoptotic role and has been implicated in the pathogenesis of several human malignancies [23]. Therefore, it may be postulated that the methylation-dependent silencing of the decoy receptors that we showed overcomes the block of NF-κB activation via *DR4* and *DR5*, thus subjecting the cancer cell to a pro-survival and pro-growth signal. Our findings indicate the need for a reappraisal of the role of the TRAIL receptors in melanoma pathogenesis and claim for a relevant impact of aberrant methylation of decoy receptors in the appearance of apoptosis evasion phenotype. Moreover, the present study may represent a starting point for new investigations which aim at clarifying the meaning and the mechanism of *DR4* and *DR5* silencing in melanoma.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.114.

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