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The RET G691S polymorphism is a germline variant in desmoplastic malignant melanoma

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Abstract

The RET protooncogene was originally identified in 1985. It encodes for a receptor tyrosine kinase. The RET receptor is activated by its ligand glial cell-derived neurotrophic factor. A polymorphism, RETp (G691S), in the intracellular juxtamembrane domain of RET, which enhances signaling by glial cell-derived neurotrophic factor has been described and studied previously in pancreatic cancer, medullary thyroid cancer, the multiple endocrine neoplasia 2 syndromes, and recently in cutaneous malignant melanoma. In particular, it has been shown that desmoplastic melanomas, which have neurotrophic features, have a high frequency of this polymorphism. In previous studies, however, it was not clear whether this was a germline or somatic change. Previous studies on pancreatic cancer indicated that both mechanisms may occur. To clarify this further we examined peripheral blood cell DNA from 30 patients with desmoplastic melanomas and 30 patients with nondesmoplastic melanoma for the RETp. In this study, a germline polymorphism was found in 30% of the patients with desmoplastic melanomas and 21% of the patients with nondesmoplastic melanoma. These findings indicate that the RETp may be a genetic risk factor for the development of desmoplastic melanoma.

Keywords

desmoplastic melanoma; G691S; germline; polymorphism; RET

Introduction

Melanoma in general often spreads to the brain, resulting in a very poor prognosis. In one of the earliest studies on melanoma and central nervous system metastasis, Amer *et al.* [1]

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Conflicts of interest

There are no conflicts of interest.

found that 75% of the patients with cutaneous melanoma had central nervous system metastasis on autopsy with a mean survival of 4 months. Recently, a large retrospective survey of almost 700 patients with brain metastases from melanoma reported a similar mean survival time (less than 5 months) [2]. Because melanocytes are derived from the neural crest during development [3], it is not surprising that brain and central nervous system metastases occur. In addition, in desmoplastic melanoma, a characteristic feature is neuronal tracking, characterized histologically by neuroma-like morphology [4].

Desmoplastic malignant melanoma is a distinct pathological and clinical subtype of the disease [5]. It is usually seen on the chronically sun-exposed sites such as the head and neck. In older individuals, desmoplastic melanomas have a higher local recurrence rate compared with nondesmoplastic melanomas and a low likelihood of regional lymph node involvement [6]. An important feature of desmoplastic melanoma is its tendency to spread along nerves [4,7] giving this subset distinctive histopathological characteristics.

The discovery of the RET protooncogene is of specific interest in desmoplastic melanoma. This tyrosine kinase receptor works with a glycosylphosphatidylinositol-anchored protein receptor (the glial cell line-derived neurotrophic factor family receptor- α family). Signaling occurs through the binding of its preferred ligand, particularly the glial cell line-derived neurotrophic factor (GDNF) [8,9]. Although expression of the RET gene in early stages of embryogenesis suggests that it may play a role in the differentiation of specific neural structures [10], most of the studies regarding RET signaling dysregulation focus on neuroendocrine tumors [11–14]. Although melanocytes are derived from the neural crest, very few studies have been carried out regarding RET signaling in primary or malignant melanoma.

An interesting single nucleotide polymorphism (SNP), G691S (RETp), located in the intracellular juxtamembrane domain encoded by exon 11 has been described. This SNP can be found both in normal individuals and in those with an increased frequency in a number of neoplasms, including medullary carcinoma of the thyroid and pancreatic cancer [15,16]. In the only study describing G691S in melanoma, Narita *et al.* [17] examined tissue samples from 70 patients with desmoplastic and 71 patients with nondesmoplastic melanoma. They found that the RETp frequency was 61% in desmoplastic melanomas compared with 31% in nondesmoplastic melanomas. Further they demonstrated *in vitro* that melanoma cells with RETp developed neurotropic features characteristic of desmoplastic melanoma. However, they did not examine the question of whether their findings were the result of a germline SNP or a somatic change in RET, as described in pancreatic cancer [16]. In the present study, we examined the frequency of the RETp using DNA from peripheral blood of patients with desmoplastic and nondesmoplastic melanomas to further clarify this question.

Materials and methods

Whole blood collected in PAX DNA tubes

Peripheral blood samples from patients with malignant melanoma were obtained from the Skin Cancer Biorepository at the University of Colorado Cancer in accordance with institutional review board approval and patient consent. Samples were identified only by a

unique biorepository number. Samples from 30 patients with nondesmoplastic melanoma and 30 patients with desmoplastic melanoma were selected randomly. Pathology reports of each patient were reviewed to confirm the diagnosis and presence of desmoplastic changes.

DNA extraction

Whole blood was collected in PAXgene DNA blood tubes and stored at 4°C until processed. Genomic DNA (gDNA) was extracted using the Blood and Tissue DNA Isolation Kit (Qiagen, Valencia, California, USA). gDNA was analyzed using the NanoDrop 1000 UV spectrophotometer (ThermoFisher, Pittsburgh, Pennsylvania, USA). gDNA samples were stored at -20°C.

Genomic DNA analysis for RETp

Exon 11 of the RET gene was amplified from approximately 100 to 300 ng of gDNA. Intronic forward and reverse primers (forward primer 5'-GACACGGCAGGCTGGAGAGC-3' and reverse 5'-TCCCTCCCTGGAAGGCAGCT-3'; Integrated DNA Technology Inc., Coralville, Iowa, USA) spanning the entire exon 11 were used for amplification, as described previously [16]. PCR was carried out using GoTaq Master Mix (Promega, Madison, Wisconsin, USA) with a final primer concentration of 1.4 µmol/l and a final reaction volume of 50 µl. Thermo-cycling was carried out on the GeneAmp PCR System (Applied Biosystems, Carlsbad, California, USA). PCR products were analyzed on a 1.5% agarose gel.

Sequencing

PCR products were purified using the ExoSap IT enzyme mix (USB Corporation, Santa Clara, California, USA). Approximately 15 ng/ml of the PCR product was sent for direct sequence analysis at the University of Colorado Cancer Center DNA Sequencing & Analysis Core. A BLAST query confirmed the presence or absence of RETp (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Statistical analysis

Differences in genomic and allelic frequencies between samples of patients with desmoplastic and nondesmoplastic patient melanomas were calculated using the Fisher's exact test for contingency tables. All statistics were done using GraphPad Prism 5.01 (GraphPad Software Inc., La Jolla, California, USA).

Results

Table 1 is a summary of the clinical characteristics in patients evaluated for the RETp. There were 30 patients in each group. Of the patients with desmoplastic melanoma, 20 were men and 10 were women. They ranged in age from 35 to 88 with a mean age of 62.7. In the nondesmoplastic group, 17 were men and 13 were women with a mean age of 53 (ranging from 24 to 84 years). With respect to tumor location, 57% of the patients in the desmoplastic group had a primary lesion on the head and neck. This is in contrast to the nondesmoplastic group, which had fewer patients with a head and neck primary (40%).

Exon 11 of the RET gene was amplified from all samples except one. This sample was determined to have nonamplifiable gDNA (data not shown). Direct sequencing confirmed the presence or absence of RET variants, as shown in Fig. 1. Figure 1 shows chromatographs for the G/G, G/A, and A/A alleles. The allelic frequency was calculated for nondesmoplastic and desmoplastic melanomas for the RETp (Table 2). In the desmoplastic melanoma group, 67% were wild-type for RET (G/G) and 30% (nine samples out of 30) were positive for the RETp variant (seven were G/A and two were A/A). In the patients with nondesmoplastic melanoma, we detected a higher number of wild-type samples (77%). Only 23% of nondesmoplastic samples tested positive for the RETp variant (six were G/A and one was A/A). This frequency was similar to that reported in the normal Caucasian population [18]. Although the difference between desmoplastic and nondesmoplastic melanoma groups did not reach statistical significance in this small patient sample, it is highly suggestive that further larger studies will bear out a difference. We also calculated the allelic frequency in desmoplastic versus nondesmoplastic melanoma. The presence of an A allele was higher in the desmoplastic group (19%) compared with the nondesmoplastic group (13%). Again this difference is not statistically significant because of our small sample size.

Discussion

In a previous study, Narita *et al.* [17] reported a high frequency of the RETp in tumor samples from 70 patients with desmoplastic melanoma (61%) compared with 71 patients with nondesmoplastic melanoma (31%). In this study, the investigators did not examine normal tissues from the same patients to determine whether the differences observed were the result of somatic changes in RET or were present as a germline SNP. In a previous study on pancreatic cancer, Sawai *et al.* [16] examined pancreatic adenocarcinomas for RETp with matched normal pancreas from the same patients. These authors found that the RETp variant could be found in 31% of the matched normal pancreatic tissue. In addition, the tumor and normal tissue did not always match indicating that RETp can be both a germline SNP and result from a somatic mutation.

In our study, we examined this question in melanoma using peripheral blood cells as a source of germline DNA. We found a high frequency of RETp as a germline SNP in desmoplastic melanoma. Although this was not statistically significant we feel that with a larger sample this finding will be confirmed. In the present study, we did not examine tissue samples from each of our patients for the polymorphism to answer the question of how often this can arise as a somatic change. These studies are underway. Initial data suggest that if RETp is present as a germline variation it is always present in the same patient's tumor. We do not, however, have enough data on concordant blood and tissue to say how often RETp may arise as a somatic variant. When viewed in light of the data from Narita *et al.* [17] it must be concluded that somatic changes can, and do arise. Our study does suggest that the presence of RETp as a germline variant may predispose individuals with this variant to the development of a specific variety of melanoma. We have also speculated that the presence of a RETp germline polymorphism might increase the probability of a family history of melanoma. While we did not find this correlation in our small sample size this is being further investigated with a larger sample size. Of note in this regard is the reported high

frequency of the RETp variant in Hispanics [18]. Although this group has a lower frequency of melanoma than non-Hispanic Caucasians it will be interesting to determine if they have a higher frequency of desmoplastic melanoma.

Regardless of the mechanism involved in the relationship between the RETp variant and development of melanoma, down regulation of the receptor may be a therapeutic tool in the treatment of patients with malignant melanoma that demonstrates the RETp. In the previous study by Narita *et al.*, they demonstrated that the presence of the polymorphism increased the response of the receptor to its ligand, GDNF. Stimulation of RETp melanoma cell lines with GDNF amplified cell proliferation, migration, and invasion; these results were not seen with RETwt melanoma cells. Similar findings were noted by Sawai and colleagues using pancreatic cancer cells. It has been suggested that the RETp pathway could be a target of small molecular weight signal transduction inhibitors including sorafenib and semaxanib [17]. In a previous study, it has been shown that sorafenib suppresses the kinase activity of RET [19]. A phase III trial in 2009 by Hauschild *et al.* [20] evaluated the efficacy and safety of sorafenib or a placebo in combination with carboplatin and paclitaxel as second-line treatment in unresectable stage III or stage IV melanomas. There were no adverse effects but the end points were unchanged compared with placebo. This trial did not, however, differentiate between desmoplastic and nondesmoplastic melanomas nor examine patients for the presence or absence of RETp. Further studies should examine the subset of patients with the RETp targeted therapy.

In conclusion, we have found that patients with desmoplastic melanoma have a higher germline frequency of the RETp than patients with nondesmoplastic melanomas or normal Caucasians. Our data suggest that the presence of this polymorphism may predispose individuals to this form of melanoma and may become a useful genetic screening tool and a target for therapy in the future.

Acknowledgements

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Fig. 1. Representative chromatographs showing G/G, G/A, and A/A genotypes (*). (a) Nondesmoplastic melanoma with G/G genotype. (b) Nondesmoplastic melanoma with G/A genotype. (c) Desmoplastic melanoma with A/A genotype.

Table 1

Clinical characteristics of melanoma patients analyzed for the RETp

Desmoplastic melanoma		Nondesmoplastic melanoma	
Number	30	Number	30
Age (year)		Age (year)	
Mean	62.7	Mean	51.5
Range	35–88	Range	24–84
Sex [number (%)]		Sex [number (%)]	
Male	20 (67)	Male	17 (57)
Female	10 (33)	Female	13 (43)
Tumor location		Tumor location	
[number (%)]		[number (%)]	
Extremity	8 (26)	Extremity	7 (23)
Head/neck	17 (57)	Head/neck	12 (40)
Torso	3 (10)	Torso	9 (30)
Unknown	2 (7)	Unknown	2 (7)

Table 2

Allelic frequency of the RETp in genomic DNA of melanoma patients

Patient classification	Genotype [number (%)]				Type of allele and allelic frequency [number (%)]		
	G/G	G/A	A/A	None (did not amplify)	No. of A alleles	No. of G alleles	None (did not amplify)
Desmoplastic (<i>n</i> = 30)	20 (67)	7 (23)	2 (7)	1 (3)	11 (18.3)	47 (78.3)	2 (3.3)
Nondesmoplastic (<i>n</i> = 30)	23 (77)	6 (20)	1 (3)	0 (0)	8 (13)	52 (87)	0 (0)