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Systematic analysis of whole exome sequencing determines RET G691S polymorphism as germline variant in melanoma

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Abstract

The RET proto-oncogene encodes a receptor tyrosine kinase that is activated by glial cell derived neutrotrophic factor (GDNF). Previous studies have found that a single nucleotide polymorphism (SNP), RETp (G691S), in the juxtamembrane domain, enhances the signaling pathway and promotes tumor growth by GDNF in pancreatic and thyroid cancer in addition to melanoma. It is uncertain however whether this SNP is a germline variant or somatic mutation. A prior study reported that the RETp variant was a germline SNP in desmoplastic and non-desmoplastic melanomas. In the present study, we examined both melanoma tissue samples and matching peripheral blood DNA to determine if RETp was 1) a germline or somatic variant, 2) more frequent in certain melanoma subtypes, and 3) frequency in brain metastasis. We examined the peripheral blood of 197 melanoma patients who had at least one matched tumor, and 42 patients with brain metastasis. RETp was present as a germline SNP in 33% of patients. There were no significant differences in RETp frequency among the different melanoma subtypes, and RETp was not correlated with brain metastasis.

Keywords

RETp, G691S, melanoma, whole exome sequencing, germline variant

Introduction

Melanoma is a unique form of cancer because of the disparity in its prognosis. When caught early there is an excellent chance of survival. However, once metastasis has occurred survival rates drop significantly despite recent advances in current molecular targeted and immune therapies.[1] The major cause of death remains brain metastasis. In an early study by Amer et al. it was shown that patients with metastatic melanoma had a 75% likelihood of brain involvement found at autopsy.[2] A recent large study of almost 700 patients diagnosed with melanoma brain metastases found a mean survival time of less than 5 months.[3] Melanoma metastasizing to the central nervous system is not unexpected considering that during development neural crest cells are the precursor cells for melanocytes, where melanoma originates.[4] Additionally, one of the common findings in melanoma is neuronal tracking, and it has been suggested that this may be related to expression of the proto-oncogene RET.[5] Plaza-Menacho et al. found that mutations that constitutively activated the RET receptor were highly related to cancers of the neuroendocrine system and of neural crest lineage.[6]

RET (REarranged during Transfection), a proto-oncogene, encodes a receptor tyrosine kinase which is activated by a glial cell line-derived neurotrophic factor (GDNF). The receptor is anchored to the cell membrane by a glycosylphosphatidylinositol-anchored protein and is expressed by many cell types including melanocytes and melanoma cells.[7,8] Interest in RET with regard to melanoma is relatively new, as it has historically been the focus of neuroendocrine related tumors. Deleterious mutations in RET have been shown to interact with other genes in Hirschsprungs disease, which affects the enteric nervous system, and in multiple endocrine neoplasia type II.[9,10] Despite melanocytes being neural crest derived, there are very few studies linking the RET proto-oncogene to melanoma.

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RETp (p.G691S) is located in the intracellular juxtamembrane domain encoded by exon 11, and may enhance the GDNF receptor-mediated cell proliferation and invasion.[7] It is commonly found in patients with medullary carcinoma of the thyroid and pancreatic cancer, but also as a germline variant in normal individuals.[7,11] Initial studies relating RETp to melanoma suggested that RETp was the result of a somatic mutation and occurs in 31% of cutaneous melanomas while present in just 15% of the general population.[12,13] This suggests that RETp places a pathogenic role in melanoma just as it does in other neuroendocrine tumors. In a subsequent melanoma study, Barr et al. showed that RETp is primarily a germline variant in desmoplastic melanoma and not a somatic mutation.[14] This raised the question whether RETp can occur in melanoma as either a polymorphism or a somatic mutation similar to findings reported in pancreatic cancer.[7] Moreover, it was also unclear as to whether this was unique to desmoplastic melanoma, or occurred in all histologic types of melanoma. To clarify these questions, we sought to determine if RETp is a germline variant in all melanoma subtypes, whether RETp is related to certain histologic subtypes of melanoma and whether there is a correlation with brain metastasis.

Materials and Methods

Sample Collection and genomic DNA isolation

Both tissue and peripheral blood samples were collected from melanoma patients at the University of Colorado Cancer Center. Samples were the stored in the International Melanoma Biorepository and Research Laboratory (IMBRL) located on the University of Colorado Anschutz Medical Campus. All collection was done in accordance with institutional review board approval and with written informed consent (COMIRB-05-0309). One hundred and ninety-seven melanoma patients with blood and tissue samples in the biorepository were examined. Many of these patients had multiple tissue or peripheral blood samples collected at different times and all samples where adequate DNA could be extracted were used as data. Sample storage, preparation and DNA isolation was performed as previously described.[13]

Whole Exome Sequencing (WES) Library Preparation and Sequencing

DNA concentration and purity was determined using Qubit (Thermo Fisher Scientific) and Agilent 2100 bioanalzyer analysis. Genomic DNA (200 ng) was sheared using Covaris S220 at 150bp. Sheared DNA was used to construct the exome library following Agilent SureSelect XT Target Enrichment System for Illumina Paired End Multiplexed Sequencing Library (cat# G9641B). Sheared DNA was end repaired followed by addition of adapter tags to construct DNA libraries through PCR amplification. Exome capture was done through hybridization using XT5 probe. Resulting captured libraries were indexed and purified. The cDNA library was validated on the Agilent 2100 Bioanalzyer using DNA-1000 chip. Libraries were sequenced on the Illumina HiSeq 2000 with 125 bp paired-end reads. We obtained an average of 400X and 200X sequencing coverage for the cancer and normal exomes, respectively.

WES and Polymerase Chain Reaction Analysis WES data analysis

WES data from patients were analyzed using IMPACT, our recently published WES analysis pipeline.[14] In brief, exome sequences were mapped to the human hg19 reference exome using

the Burrows-Wheeler Aligner (BWA) (v0.7.8-r455).[16] SAMTools (v1.1)[17] and BCFtools (v1.1) were utilized to generate a variant call format (VCF) file that was annotated by ANNOVAR (v2014-11-12).[18] A minimum of 20 reads had to occur for a variant to be called. Of those 20 reads at least 4 reads needed to be mutated, or 10% of all reads needed to be mutated. From the VCF file, we compared the paired-normal and tumor samples to determine germline or somatic RETp variant.

PCR Sequencing

An additional 49 patient peripheral blood samples with desmoplastic melanoma that did not have matched tumor samples were analyzed with PCR and Direct Sanger Sequencing. The methods used for PCR sequencing were previously described by Barr et al and were used to determine the RETp genotypes of samples that had not undergone WES.[13]

The Cancer Genome Atlas (TCGA) Skin Cutaneous Melanoma Samples

TCGA is a joint effort between the National Cancer Institute and the National Human Genome Research Institute to track genomic changes in many types of cancer. In order to increase the power of the study, we obtained the germline and somatic variants of 331 cutaneous melanoma patients from the TCGA Data Portal (https://tcga-data.nci.nih.gov/).[19] From the SNP variant call format (VCF), we extracted the RETp (rs1799939) from these patients. RETp variant from these patients were classified as germline or somatic based on the Broad Institute Automatic Mutation Analysis.

Statistical analysis

Genotypes of each sample were determined and a two-tailed Fisher's exact test was done to determine differences between groups of samples. A hypergeometric test was performed to determine the difference between molecular subtypes. $P < 0.05$ was considered as statistically significant.

Results

Whole exome sequencing of 197 melanoma patients across multiple subtypes was assessed by next-generation sequencing. On average, we obtained 400X coverage for cancer and 200X coverage for normal exomes. The mapping rate for these exomes was 94%. To evaluate whether RETp is a germline or somatic variant, we compared the paired normal and tumor exomes in 197 melanoma patients. We found the RETp variant in 65 patients where 62 (95%) and 3 (5%) were germline and somatic variants, respectively (**Figure 1A**). Similarly, 107 of the 331 (32%) TCGA cutaneous melanoma patients carried this RETp variant. Among the 107 TCGA melanoma patients with RETp, 103 (96%) and 4 (4%) were germline and somatic variant, respectively (**Figure 1B**). From both data sets, we can conclude that RETp is a germline variant in melanoma.

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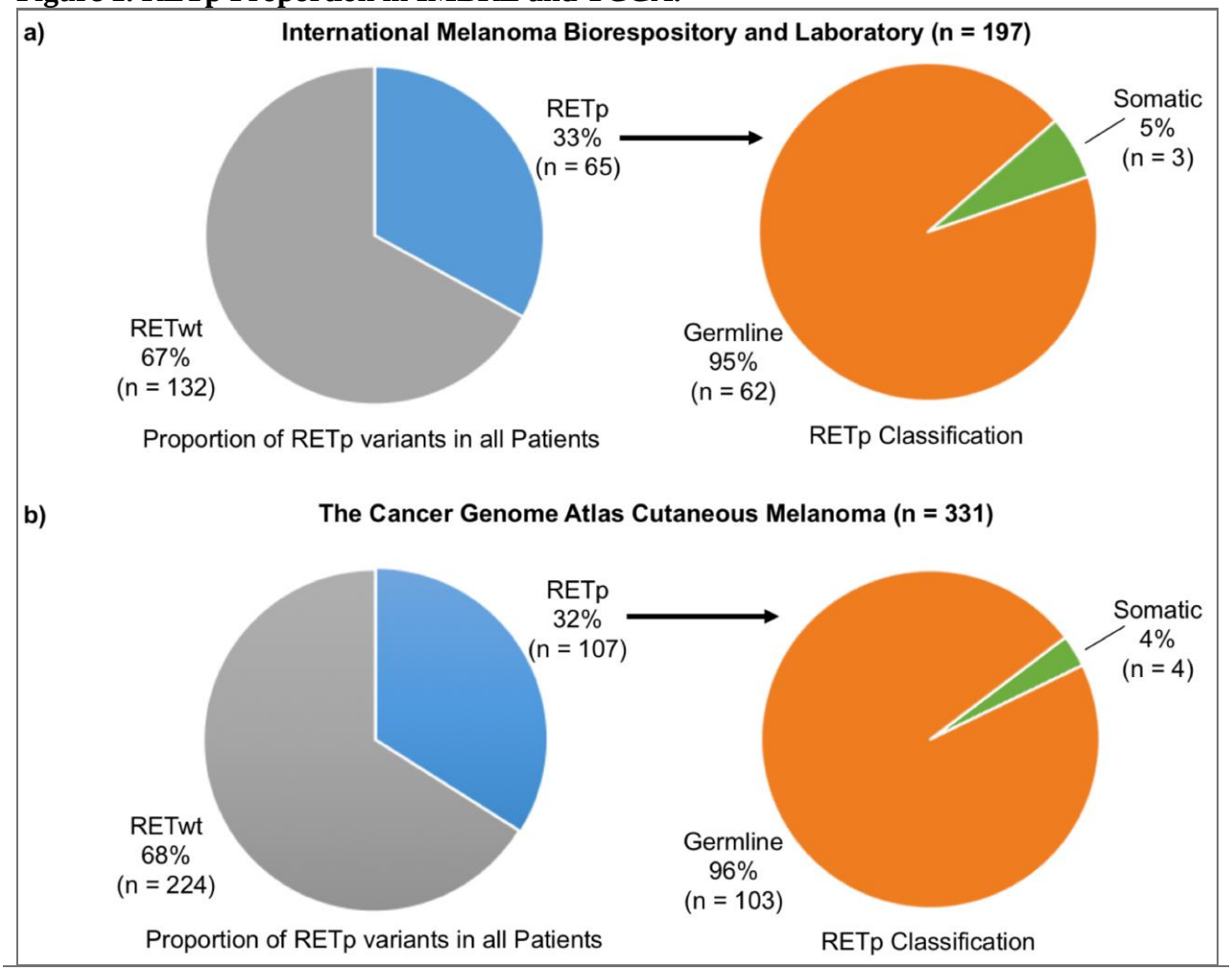
Figure 1. RETp Proportion in IMBRL and TCGA.



Proportion of patients with RETp variant (germline or somatic) and RETwt wildtype in IMBRL (a) and TCGA (b) datasets.

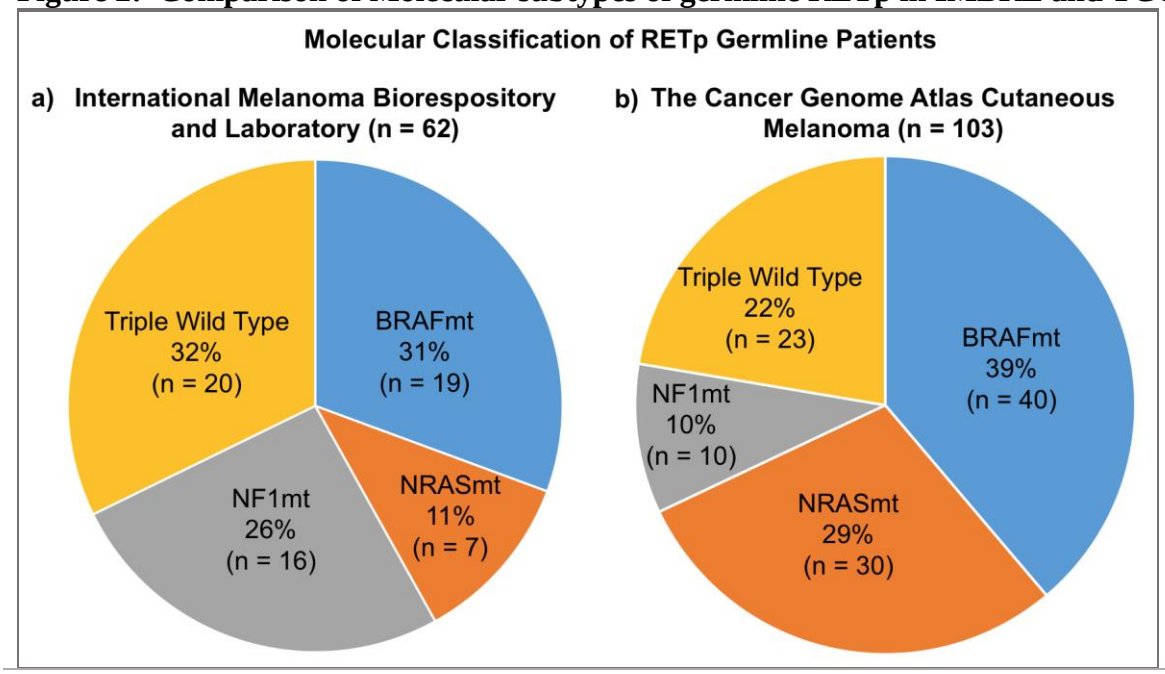
To assess whether RETp is more frequent in certain melanoma subtypes, we evaluated the germline RETp variant across six melanoma subtypes in the peripheral blood of 197 WES patient's and 49 desmoplastic patients (acral lentiginous, mucosal, nodular, superficial spreading, unknown primary, and desmoplastic). We found that on average, 33% of the 246 patients carried a RETp germline variant (**Table 1**) in these subtypes, with range from 29 – 42%. In this study, we found that the nodular subtype had the highest percentage of the RETp germline variant (42%, p-value = 0.27) among the other subtypes. There were no significant differences between RETp and RETwt frequency in any of the melanoma subtypes. Similarly, there was no significant difference in the frequency of RETp between melanoma subtypes. The RETp germline variant in our study is similar to the TCGA cutaneous melanoma samples (32%, 107 / 331 RETp germline variant). This analysis strengthens the hypothesis that RETp is a germline variant in melanoma, and is not enriched in specific histological subtypes of melanoma.

Table 1: Frequency of RETp germline across melanoma subtypes.

Melanoma Subtype	Wild-type RET	RETp germline	Total (% RETp)	p-value
Acral Lentiginous	9	5	14 (36)	0.7754
Mucosal	9	4	13 (31)	1.0000
Nodular	15	11	26 (42)	0.2739
Superficial Spreading	43	17	60 (28)	0.5264
Desmoplastic	36	19	55 (35)	0.7452
Unknown Primary	8	5	13 (38)	0.7619
Other	46	19	65 (29)	0.5408
Total	**166**	**80**	**246 (33)**	

Next, we asked whether germline RETp is correlated with different molecular subtypes of melanoma in the cohort of 197 patients that were analyzed by WES. Here, we followed the four molecular subtypes of melanoma defined by: BRAF-mutant, NRAS-mutant, NF1-mutant and triple wild-type.[19] In our 62 melanoma patient samples with germline RETp, 31%, 11%, 26% and 32% were BRAF-mutant, NRAS-mutant, NF1-mutant and triple wild-type subtypes, respectively (**Figure 2a**). Similarly, in the TCGA patients with the RETp germline variant there were 39%, 29%, 10%, and 22% RETp in the BRAF-mutant, NRAS-mutant, NF1-mutant and triple wild-type subtypes, respectively (**Figure 2b**). A hypergeometric test concluded there was no enrichment of RETp in any of these molecular subtypes in either cohort (p-value > 0.5). S1 Table lists out the distribution of the germline RETp variant in these molecular subtypes for our cohort and the TCGA cutaneous melanoma cohort.

Figure 2: Comparison of Molecular subtypes of germline RETp in IMBRL and TCGA.



Molecular Subtypes of melanoma (BRAF-mutant, NRAS-mutant, NF1-mutant and triple wild-type) with germline RETp in IMBRL (a) and TCGA (b) samples.

We next asked whether germline RETp is correlated with brain metastasis in melanoma. We assessed germline RETp variant of 42 brain metastasis and 90 other metastasis patients in this study. We found that germline RETp variant was present in 33% of melanoma patients with brain metastasis and 31% of patients with other metastasis (p = 0.84) (**Table 2**). Additionally, we assessed whether germline RETp variant is correlated with primary vs metastatic melanoma. We found no enrichment of germline RETp variant with primary vs. metastases in our cohort as well as the TCGA cutaneous melanoma cohort (**Table 2**).

Table 2. Frequency of germline RETp variant in melanoma patients with brain and other metastasis.

	Brain Metastasis	**Other Metastasis**	**p-value**
RET wildtype	28	62	
Germline RETp	14	28	
Total (% RETp)	**42 (33)**	**90 (31)**	**0.84**

Discussion

In this study, we performed a systematic analysis of RETp variant in melanoma using whole exome sequencing data. We demonstrated that RETp variant was found to be present in 33% of our 197 melanoma patients. This frequency is similar to the 331 TCGA cutaneous melanoma samples (32%). As one of our main objectives of this study was to determine whether RETp is a germline or a somatic variant, we assessed paired normal and matched melanoma patients in our data. We found that in 95% of the patients studied here RETp was germline, and this was supported by the same findings in TCGA cutaneous melanoma patients (96% of the RETp is germline variant). Based on these two large studies, we conclude that RETp is a germline variant in melanoma patients. This also agrees with our previous study involving desmoplastic melanoma and RETp.[14] This study is also an important example of why using both tumor and blood samples is essential to calling somatic variants. Tumor-only analysis has the potential to incorrectly identify RETp as a somatic variant.

To assess the frequency of germline RETp across melanoma subtypes, we evaluated this variant in acral lentiginous, mucosal, nodular, superficial spreading, unknown primary, and desmoplastic. From our data, we did not observe any association between germline RETp with melanoma subtypes. In a limited number of available samples, acral melanomas had a higher incidence of the RETp variant. This subtype arises on glabrous skin which may account for the differences seen, however more samples are needed. Similarly, we did not find any association of germline RETp with molecular subtypes of melanoma.

Cutaneous melanoma can be classified into one of four molecular classifications based on the presence of hotspot mutations in BRAF or NRAS, deleterious mutations in NF1, or a lack of mutations in these three genes (triple wild type).[18] We analyzed the presence of the RETp germline variant in each molecular classification for both cohorts of samples. There was no enrichment of RETp in any of the four molecular classifications in either cohort of samples (**Table 1**).

To evaluate whether germline RETp is correlated with brain metastasis, we compared 42 brain metastasis and 90 samples from other metastatic sites. The percentage of germline RETp variant is 33% and 31% in brain metastasis and other metastasis, respectively. We did not find that brain metastasis is enriched for germline RETp. We also evaluated the presence of RETp in primary and metastatic sites in our cohort as well as the TCGA samples. We found no association between the presence of RETp and primary or metastatic sites in either cohort (**Table 2**).

In one of the studies done here, there was discordance between the blood and tumor tissue for RETp. There are multiple explanations for these findings including allelic loss and perhaps somatic mutation in tumor. These variants were not associated with any clinical scenario or other molecular events and have not been pursued further at this point.

Despite the findings here multiple studies have shown the importance of alteration of RET in other cancers, particularly medullary and papillary carcinoma of the thyroid.[20] In these cancers, rearrangements, point mutations, deletions and insertions have been demonstrated which may occur as both hereditary and somatic changes. While it was initially thought that many of these were exclusive to thyroid cancers, similar alterations have been then described in other cancers, but not malignant melanoma. We think that further studies in melanoma will however turn up similar activating alterations in RET and these are currently underway in our laboratory.

Conclusion

In summary, we have performed a systematic analysis of RETp variant in a large cohort of melanoma patients. By assessing the whole exome sequencing of these melanoma patients, we identified 33% and 32% of patient samples had RETp variants in our samples and TCGA cutaneous melanomas, respectively. On average, 96% of the RETp variant is germline in our samples and TCGA samples. There was no association of germline RETp with melanoma subtypes and molecular subtypes. Furthermore, we did not find any enrichment of germline RETp in brain metastasis melanoma patients.

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