RESEARCH PAPER

Cancer Biology & Therapy 10:1, 33-37; July 1, 2010; © 2010 Landes Bioscience

Novel somatic mutations in heterotrimeric G proteins in melanoma

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Key words: heterotrimeric G proteins, GNG10, GNAZ, melanoma

Heterotrimeric guanine nucleotide-binding proteins (G proteins) mediate signals between G-protein coupled receptors and their downstream pathways, and have been shown to be mutated in cancer. In particular, GNAQ was found to be frequently mutated in blue nevi of the skin and uveal melanoma, acting as an oncogene in its mutated form. To further examine the role of heterotrimeric G proteins in melanoma, we performed a comprehensive mutational analysis of the 35 genes in the heterotrimeric G protein gene family in a panel of 80 melanoma samples. Somatic alterations in a G protein subunit were detected in 17% of samples spanning seven genes. The highest rates of somatic, non-synonymous mutations were found in GNG10 and GNAZ, neither of which has been previously reported to be mutated in melanoma. Our study is the first systematic analysis of the heterotrimeric G proteins in melanoma and indicates that multiple mutated heterotrimeric G proteins may be involved in melanoma progression.

Introduction

Heterotrimeric guanine nucleotide-binding proteins (G proteins) mediate signals from transmembrane G protein coupled receptors (GPCRs) resulting in a variety of cell responses.[1,2] Activation of G proteins has been shown to impact several signaling pathways including those involving mitogen-activated protein kinases (MAPKs) as well as other signaling pathways affecting oncogenesis and metastasis.[2-5]

Recently, somatic mutations in the G protein alpha subunit q (GNAQ) were shown to be frequently mutated in blue nevi of the skin and uveal melanoma.[6] These mutations were found to occur in the ras-like domain and result in constitutive activation of the MAPK pathway.[6] In order to test whether GNAQ as well as the rest of the heterotrimeric G protein superfamily are somatically mutated in melanoma, we used high-throughput gene sequencing as a systematic genetic profiling approach.

Results

Activation of G proteins is known to impact cellular processes such as growth, proliferation, adhesion, migration and invasion through a variety of pathways.[2-5] Recent literature suggests that GNAQ is an oncogene in human neoplasia in melanoma, behaving similarly to melanocytic mutations in BRAF or NRAS (Lamba et al., 2009; Van Raamsdonk et al. 2009). To further explore the potential role of G proteins in melanoma progression, we sequenced the entire G protein gene family in a panel of 80 metastatic melanoma tumors (**Suppl. Table 1**). Exon specific primers were designed to amplify and sequence the entire coding region (**Suppl. Table 2**). A total of 37 exons, spanning over 638 kb of genomic tumor DNA, as well as at least 15 intronic bases at both the 5' and 3' ends, were directly sequenced. 100% of the 37 exons amplified were successfully sequenced.

Initially we sequenced all of the coding exons in a sub-set of 32 malignant melanoma tumors from our tumor bank. All potential mutations found in tumor tissues were then re-sequenced in parallel with matched normal tissue to confirm that mutations were somatic. During this process, we identified 7 genes to have at least 1 non-synonymous somatic (tumor-specific) mutation. Next, we followed up on genes which were found to have somatic mutations in the tumor sample by sequencing them in the remaining panel of 48 tumors. We also sequenced these 7 genes in 12 commercially available melanoma cell lines.

From the mutational analysis of the melanoma tumor bank, a total of 18 non-synonymous and 3 synonymous somatic mutations were identified in 7 genes (**Table 1**). The overall non-synonymous somatic mutation rate in melanoma was determined to be 17.5%. Of the 7 genes with non-synonymous somatic mutations, 6 genes (GNA12, GNG10, GNA14, GNA15, GNA16 and GNB3) have not previously been shown to be mutated in

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Submitted: 02/18/10; Revised: 03/31/10; Accepted: 04/04/10
Previously published online: www.landesbioscience.com/journals/cbt/article/11949
DOI: 10.4161/cbt.10.1.11949

Table 1. Nonsynonymous somatic mutations identified in heterotrimeric G proteins

Gene	Other names	CCDS accession*	Ref Seq accession*	No. of mutations (% tumors affected)#	Tumor	Exon	Nucleotide†	Amino Acid†	Functional domain‡	NRAS/BRAF mutation**
GNA12	MGC104623 MGC99644 NNX3 RMP WUGSC:H_GS165O14.2 gep	5335.1	NM_007353	1 (1.25)	13T	2	C353T	P118L	N/A	None
GNG10	GNGT10	43862.1	NM_004125	4 (5.0)	76T	1	C23T	P8L (LOH)	G protein gamma	*BRAF*
	OTTHUMP00000021914				39T	1	A155C	E52A	G protein gamma	None
	OTTHUMP00000063926				13T	3	C643T	R215C	N/A	None
	DNAJC25-GNG10				34T	4	G1038A	W346X	N/A	*BRAF*
GNAZ	Gz-alpha	13804.1	NM_002073	4 (5.0)	24T	1	A128G	N43S	G protein alpha	*NRAS*
					32T	1	C239T	S80L	G protein alpha	*BRAF*
					4T	1	C292T	P98S	G protein alpha	*BRAF*
					18T	1	G451A	D151N	G protein alpha	None
GNA14	OTTHUMP00000021515	6657.1	NM_004297	4 (3.75)	62T	1	T74A	L25H	G protein alpha	None
					13T	3	G310A	E104K	G protein alpha	None
					105T	4 / 7	G536A / C1000T	R179Q / R334C	G protein alpha /G protein alpha	*BRAF*
GNA15	GNA16	12104.1	NM_002068	3 (3.75)	96T	4	G517A	E173K	G protein alpha	None
					52T	7	C901T	P301S	G protein alpha	*BRAF*
					18T	7	C1096T	R366C	G protein alpha	None
GNA11	GA11	12103.1	NM_002067	1 (1.25)	45T	5	G667A	V223M	G protein alpha	*BRAF*
GNB3	N/A	8564.1	NM_002075	1 (1.25)	17T	5	C293T	S98F	WD40	*NRAS*

*Accession numbers for mutated G proteins in Santa Cruz and GenBank. #Number of non-synonymous and splice site mutations observed and percent of tumors affected for each of the 7 genes in the panel of 80 melanoma cancers. †Nucleotide and amino acid change resulting from mutation. When two mutations in the same gene in a tumor were observed, the mutations are separated by a slash. "X" refers to stop codon. "LOH" refers to cases wherein the wild-type allele was lost and only the mutant allele remained. **Mutations previously observed in NRAS, BRAF. "None" refers no mutation observed. ‡Abbreviations for the functional domains: G protein alpha: G protein alpha subunit; G protein gamma: G protein gamma subunit-like motif.

any tumor type: (www.sanger.ac.uk/genetics/CGP/Census). GNA11 was previously reported to have a mutation rate of 17% (1/6) in malignant melanoma (www.sanger.ac.uk/genetics/CGP/Census); our analysis reflected a much lower mutation rate: it was mutated in only 1/80 (1.25%) samples. Another study, which only sequenced exon 5 of GNA11, did not find any mutations in the 922 tumor samples examined, including 24 melanomas.[7] As our study examined the whole of the gene for somatic mutations, this difference may be due to the limited number of exons examined in the Lamba et al. study (which only looked at exon 5). GNAZ was also previously reported to be mutated in 1/101 clear cell renal cell carcinoma samples (www.sanger.ac.uk/genetics/CGP/Census), but has not previously been shown to be mutated in melanoma. The genetics of clear cell renal cell carcinoma are known to be distinctive; cancer genes which are frequently mutated in other adult epithelial cancers, are rarely mutated in clear cell renal carcinoma,[8] which may account for the difference in mutation rates between the two tumor types.

Full sequencing of the exons and splice sites of these 7 G protein genes resulted in a NS:S mutation ratio of 18:3, higher than the expected non-selected passenger mutation 2:1 NS:S ratio ($p < 0.01$). Furthermore, there was a significant ratio of C:G>T:A mutations compared to other nucleotide substitutions, consistent with known melanoma mutation signatures ($p < 0.02$) (Greenman, et al. 2007) (**Fig. 1**). Many tumors with G protein mutations were also found have mutations in NRAS or BRAF (**Table 1**). The clinical information associated with the melanoma tumors containing somatic G protein mutations is provided in **Supplementary Table 3**.

Non-synonymous mutations were also found in 7 of 12 commercially available melanoma lines for which these 7 G protein genes were analyzed (**Suppl. Table 4**). In four of the genes, GNG10, GNA14, GNA11 and GNB3, the mutations found in the commercially available melanoma lines were in the same functional domain as a mutation which was found in our melanoma tumor bank.

To test whether the two most highly mutated genes, GNAZ and GNG10 are also altered by amplification, quantitative PCR of these two genes was performed in the same set of 80 melanoma samples. No amplifications were observed for GNAZ (data not shown), however, three out of the 80 samples showed amplification of GNG10 (**Fig. 2**). No GNG10 mutations were present in these three samples, demonstrating that GNG10 mutations and amplifications occurred in a mutually exclusive pattern. Taken together, these results showed that GNG10 was somatically altered in 8.75% (7/80) of melanoma metastases. The combination of the somatic mutations and amplifications identified in GNG10 warrant functional analysis to assess the possibility that GNG10 has a role in melanoma tumorigenesis.

Discussion

The two genes with the highest somatic mutation rate were GNG10 and GNAZ (**Suppl. Fig. 1**); these genes were each found to have non-synonymous somatic mutations in 4/80 (5%) tumors. The four novel mutations in GNAZ were all found to be in the G protein alpha subunit domain; they affect amino acids residues that are highly conserved across species (**Suppl. Fig. 2**). The alpha subunit is an important component of G protein interaction with receptors and effectors, and contains the guanine nucleotide binding site and regulates the kinetics of signaling through an intrinsic GTPase activity. Therefore, mutations in the G protein alpha subunit domain in GNAZ may affect critical functions of this gene.

GNAZ is distinct from other G-alpha genes; its relatively low sequence homology (60%) with the other members of the G_i protein subfamily is reflected in its unique biochemical properties within the subfamily.[9] G-alpha z is subjected to a variety of covalent modifications, and hydrolyzes GTP with slower kinetics than the other G-alpha subunits.[10] Although the relationship between G-alpha z and the MAPK signaling pathway is not yet fully understood, there is evidence that G-alpha z might contribute to the regulation of specific subfamilies of MAPKs, including extracellular signal-regulated kinases and Jun N-terminal kinases.[11,12] Previous biochemical studies on GNAZ elucidating these relationships have not been conducted in cancer cells; however, as these signaling pathways are known to be important in cancer progression, future studies examining the impact of mutations in GNAZ in tumor cells are warranted.

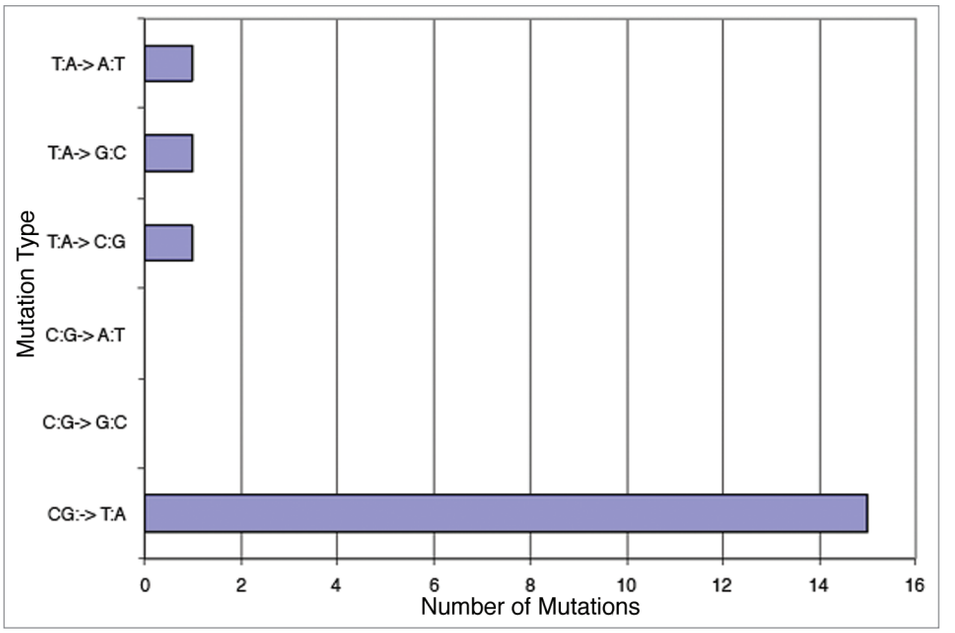

Figure 1. Mutation spectra of single base pair substitutions in heterotrimeric G proteins. The number of each of the six classes of base substitutions resulting in non-synonymous changes in heterotrimeric G proteins is shown.

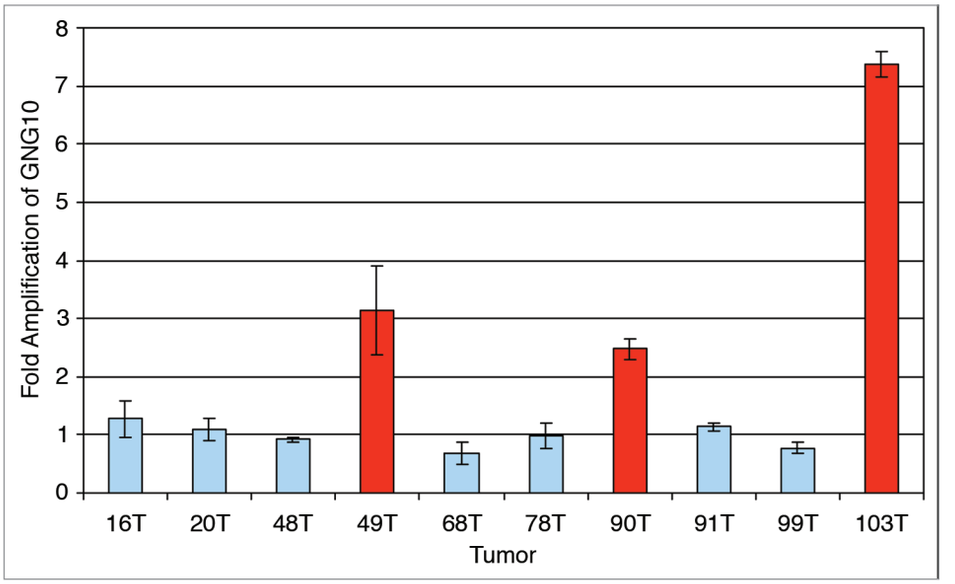

Figure 2. GNG10 is amplified in melanoma in 49T, 90T and 103T. Genomic DNA from 80 metastatic melanoma tumors was analyzed by real-time PCR for copy number of GNG10. Amplification is expressed as fold-increase in signal for GNG10 after normalization to an internal control gene LINE1. Error bars represent standard deviation between two replicates.

Of the four somatic mutations in GNG10, only two fall into the G protein gamma subunit-like domain. These two missense mutations found in GNG10 do not display high levels of evolutionary conservation; however, both p.P8L and p.E52A result in a reverse in charge of the amino acid. The change from hydrophilic to hydrophobic may have particular functional importance for p.E52A as the G protein gamma subunit is known to have hydrophobic interactions with the G protein beta subunit at p.D51 and p.L53.[13]

We detected one NS somatic mutation in GNA12. Several previous studies have begun to elucidate the importance and functional roles of GNA12 in cancer development and progression. Members of the G12 family, which includes G-alpha 12 and 13, were found to promote the growth and oncogenic

transformation of murine fibroblasts.[14,15] Kelley et al. have shown increased expression of G-alpha 12 in breast cancer, and that signaling through the G12 family of G proteins promotes breast cancer cell invasion, and that inhibition of G12 signaling reduces breast cancer metastases in vivo.[16] A follow-up study by the same group showed that G12 family proteins were also regulators of prostate cancer invasion, and blocking the G12 signaling pathways reduced cell invasion in vitro.[17] GNA12 has also previously been shown to be differentially expressed between oral squamous cell carcinoma and normal oral mucosa, suggesting that it may play a role in oral squamous cell carcinoma carcinogenesis.[18]

We identified two novel changes in GNB3. GNB3 and its pathways have also been shown to be functionally important in cancer progression. Previous studies have shown that a C825T polymorphism results in a splice variant with a deletion of 41 amino acids and increased G protein activation. This polymorphism has been associated with oncogenesis and increased metastases in a variety of cancers, including breast, colorectal, head and neck squamous cell, and thyroid cancers.[19-23] The results of these studies, combined with our data showing a mutation in malignant melanoma in GNB3, suggest that further examination of pathway is warranted.

GNA11 has previously been sequenced in melanoma by two different groups. One study reported a mutation rate of 29% (2/7) in malignant melanoma (www.sanger.ac.uk/genetics/CGP/Census); the second study sequenced only exon 5 of GNA11, but found no mutations in the 24 melanomas examined.[7] Although we only found one somatic mutation in GNA11, this gene is of great interest phenotypically as it has previously been shown to have reduced expression in breast cancer.[24] mRNA expression was found to be decreased in 62.5% (10/16) of human breast cancers by RT-PCR, and the immunoreactivity of the G-alpha 11 protein reduced in 14 of 16 cancers.[24] This study by Asada et al. suggests that reduced G-alpha 11 levels are advantageous for the growth of breast cancer cells; functional work on GNA11 could help elucidate the mechanisms behind this phenotype.

GNA14 and GNA15 are both members of the G_q class of G-alpha proteins, along with GNA11, GNA16 and GNAQ. Although few functional studies have been conducted on these two genes, it is of note that we found mutations in 3 of the 5 members of this class: GNA11, GNA14 and GNA15. This class of G proteins is largely involved in inositol lipid signaling through the activation of beta-isoforms of phospholipase C.[25] Receptors coupled to the G_q class of G-alpha proteins mediate a wide range of cellular responses, including cell growth and proliferation, glucose secretion, and leukocyte activation.[25] The unique distribution patterns of the members of this class of proteins suggests unique signaling functions, particularly for G-alpha 14 and 15, which have a more limited distribution pattern than the ubiquitously expressed G-alpha 11.[25] Combined, this data suggests that each of these genes is worth studying individually to determine its role in melanoma development and progression.

Interestingly, our results showed that there were no GNAQ mutations in any of the tumors. This result is consistent with previously published studies showing that GNAQ has a high mutation rate in blue naevi and uveal melanoma, but not other types of melanoma.[6,7] In melanoma subtypes with no H-, N-, K-RAS or BRAF mutations, GNAQ has been shown to act as an oncogene. The lack of mutations in GNAQ found in this study supports the conclusion that GNAQ is a critical cancer gene in blue naevi and uveal melanoma, but not metastatic melanoma.

In conclusion, we have completed the first systematic genetic analysis of the heterotrimeric G protein gene family in melanoma. This lead to the identification of 18 non-synonymous somatic mutations in 7 different genes in melanoma; 5 of which have not previously been reported to be mutated in cancer. The NS:S mutation ratio, which was significantly higher than the expected non selected passenger mutation ratio, suggest that mutated G proteins could play a functional role in melanoma progression.

Materials and Methods

Tissue samples. A panel of pathology-confirmed metastatic melanoma tumor resections, paired with apheresis-collected peripheral blood mononuclear cells, was collected from 80 patients at the Surgery Branch of the National Cancer Institute, with informed consent from all human subjects, as previously described.[26]

PCR, sequencing and mutational analysis. Polymerase chain reaction amplification, sequencing and analysis was performed as previously described.[27,28] Briefly, the initial screen of 32 tumors was analyzed using Consed,[29] and variants were called using Polyphred 6.11.[30] Sequences traces of the follow-up screen of 48 tumors were analyzed using the Mutation Surveyor software package (SoftGenetics). Additionally, 12 commercially available cell lines, which have no matched-normal gDNA, were analyzed for mutations.

Quantitative real-time PCR of genomic DNA for copy number analysis. Gene copy number for GNG10 was determined by quantitative RT-PCR (iCycler, Bio-Rad, Hercules, CA) of genomic DNA from 80 tumors and quantified by comparing the GNG10 locus to the reference locus LINE1. PCR conditions were as follows: 15.2 µL double-distilled water (Invitrogen, Carlsbad, CA), 2.5 µL of 10X PCR buffer (670 mM Tris-HCl [8.8], 67 mM $MgCl_2$, 166 mM $(NH_4)_2SO_4$ and 100 mM 2-mercaptoethanol), 2.5 µL of 10 mM deoxynucleotide triphosphates (Promega, Madison, WI), 15 µL of DMSO (Sigma, St. Louis, MO), 0.5 µL of SYBR Green I solution (Invitrogen, Carlsbad, CA) diluted 1:1,000 in double-distilled water, 0.5 µL of 50 µM forward and reverse primers (IDT), 0.25 µL of Platinum Taq DNA polymerase (Invitrogen), and 2 µL of purified genomic DNA. Reactions were cycled at: (94°C, 2 min) x1, (94°C, 10 s; 57°C, 15 s; 70°C, 15 s) x35. PCRs were done in duplicate using primers in **Supplementary Table 1**. The GNG10 gene amplification in tumor DNA (A_{Tu}) was determined using the formula $A_{Tu} = 2^{-\Delta CT}$ with ΔCT being [C_T (GNG10;tumor) - C_T (LINE1;tumor)].

Statistics. The statistical significance of the non-synonymous (NS): synonymous (S) ratio of observed mutations was calculated with the exact binomial test compared to a 2:1 NS:S ratio (Sjoblom, et al. 2006). All the statistical calculations were performed in the R statistical environment (www.r-project.org).

Acknowledgements

We thank members of the NISC Comparative Sequencing Program for providing leadership in the generation of the sequence data analyzed here. Funded by the National Human Genome Research Institute and National Cancer Institute, National Institutes of Health.

Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/NaviaCBT10-1-Sup.pdf

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