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Molecular studies of melanoma

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INTRODUCTION

The etiology of cutaneous melanoma is incompletely understood although genetic alterations together with the exposure to solar ultraviolet radiation are major risk factors (1). Growing evidence suggests that the development and progression of melanoma is dependent, in part, on activating mutations in genes such as *RAS* and *RAF* that regulate the mitogen-activated protein (MAP) kinase signal transduction pathway (2,3). Recent studies suggest that activating mutations in *NRAS* are present in 15-30% of melanoma samples (4-6) in contrast to *HRAS* and *KRAS*, which are rarely mutated. These are predominantly the missense mutations in codons 12 and 13 of the exon 1 and codon 61 of the exon 2 of *NRAS* gene and are thought to result in constitutive activation of the MAP kinase pathway (3,7). Furthermore, 95% of familial melanoma tumors in individuals with germline *CDKN2A* mutations have been identified as carrying such activating *NRAS* mutations (8). *BRAF* mutations have been observed in up to 60-70% of melanomas (9-12), 80% of which are identical mutations in codon 600 (previously codon 599) (10). Similarly, to mutated *NRAS*, mutated *BRAF* encodes a protein with constitutive serine/threonine kinase activity that is capable of causing hyperstimulation of the MAP kinase cascade (13). Interestingly, less than 1% of melanoma patients are found to carry a mutation in both genes (10,12). Furthermore, the primary tumors as a rule carry *NRAS* and *BRAF* mutations identical to those in the metastases derived from the primary tumor (12).

The identification of genes differentially expressed in cells containing activating mutations in either *NRAS* or *BRAF* may reveal molecular targets that can enhance the development of treatments with higher efficacies for suppressing the MAP kinase pathway. Thus, intracellular signaling may differ in melanoma tumors with different *NRAS*/*BRAF* mutation status.

The aim of these studies therefore were to identify the frequency of *NRAS* and *BRAF* mutations in primary melanomas, in relation to the overall survival, as well as to compare the gene expression profiles of metastatic melanoma samples in order to identify differentially expressed genes that distinguish tumors with either *NRAS* or *BRAF* mutations from tumors without such mutations. Here we present preliminary results of ongoing projects.

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MATERIAL AND METHODS

The Ethics Committee of the Karolinska Institute approved these studies.

Primary melanoma study

Cohort

Forty-one melanoma patients were separated into two groups with respect to their 5-year overall survival; 29 survived more than 5 years and 12 survived less than 5 years.

DNA extraction

Four serial sections (6 μm) were cut from each paraffin-embedded biopsy sample, and one of the inner sections was counterstained with hematoxylin and eosin as a guide for laser-capture microscope (LCM). The sections were then microdissected with a PixCell LCM (Arcturus Engineering, Mountain View, CA). The dissected sections (50-200 cells) were incubated in 30-50 μL (depending on the number of cells in the target area) of lysis buffer (proteinase K at 1 mg/mL [Sigma-Aldrich, Steinheim, Germany] and 1% Tween 20 in TE buffer [10 mM Tris-HCl, 1 mM EDTA; pH 8.0]) at 56˚C overnight. Proteinase K was inactivated by incubating the samples in lysis buffer at 95˚C for 10 minutes.

For use as positive controls for the exon 2 of the *NRAS* gene, DNA was also extracted from the human HT1080 fibrosarcoma and 224 metastatic melanoma cell lines by freeze-thaw incubations and proteinase K treatment, followed by DNA purification protocol using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI), according to the manufacturer's instructions. The HT1080 cell line carries an AAA (Lys) substitution, and the 224 metastatic melanoma cell line carries a CGA (Arg) substitution at codon 61 of the *NRAS* gene.

Extracted DNA from the human A375 metastatic melanoma cell line was used as positive control for the exon 15 of the *BRAF* gene (same protocol as outlined above). The A375 cell line carries a GAG (Glu) substitution at codon 600 of the *BRAF* gene. The human melanoma cell line A375 was purchased from the American Type Culture Collection (Manassas, UA).

Mutation analysis

For SSCP analysis, the PCR products were denatured in denaturing buffer at 92˚C for 10 min. Electrophoresis was carried out on 7.5% nondenaturing acrylamide gels with 10% glycerol at 18˚C, for the *NRAS* mutation detection, and without glycerol at 5˚C for the *BRAF* mutation detection. Shifted bands were cut out from the gels, reamplified, and purified using the QIAquick Gel Extraction kit (Qiagen, Hilden, Germany). Nucleotide sequence analyses were carried out using the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Sequence analyses were performed in both directions, using the nested primers as sequencing primers. Mutations were confirmed by two independent PCR/SSCP analyses.

Gene expression profiling study

Cohort

Patients (n=57) were randomly selected for this study with 5 patients with two metastatic samples, which resulted in a total of 62 metastatic melanoma samples for analysis. Overall, 23 melanomas harbored mutations in codon 61 of *NRAS*, 25 melanomas harbored mutations in codon 600 (previously 599) of the *BRAF* gene and 14 melanomas were wild type (wt) for the screened exons of *NRAS* and *BRAF*.

RNA Preparation

Total cellular RNA was then isolated from the fresh frozen melanoma samples using an RNeasy® mini RNA extraction kit (Qiagen, GmbH, Hilden, Germany), as per the manufacturer's directions.

Microarray Profiling

Preparation of *in vitro* transcription products from the 62 human melanoma samples, oligonucleotide array hybridization and scanning were performed for Affymetrix high density oligonucleotide array HG-U133 A chips, which contain

22 215 probe sets representing 18 400 transcripts and variants, including 14 500 well-characterized human genes (Affymetrix. Affymetrix Genechip™ Technical Manual, 2001).

Data Analysis

Pre-processing of the expression data was performed using GeneSpring® 6 software (Silicon Genetics) that resulted in a final of 14 635 probe sets for further analysis. A Welch ANOVA test was performed to identify genes differentially expressed in the 3 genetic classes of melanoma, i.e. (1) wt for both genes; (2) *NRAS* mutation; and (3) *BRAF* mutation. We identified 1 098 probe sets to be differentially expressed in the 3 defined classes with a p-value < 0.05. Supervised hierarchical cluster analysis was applied to the 62 human melanoma samples using the 1 098 differentially expressed probe sets in the three different genetic variants of melanoma with a p-value < 0.05.

RESULTS

Primary melanoma study

The overall frequency of the *NRAS* mutations for both exons was 6 in 41 i.e. 15% (Table 1).

Table 1. *NRAS* and *BRAF* mutation frequency

	NRAS exon 1	*NRAS* exon 2	*BRAF* exon 15
Lived more than 5 years	1/29 (3%)	2/29 (7%)	8/29 (28%)
Lived less than 5 years	0/12 (0%)	3/12 (25%)	8/12 (67%)
Total	1/41 (2%)	5/41 (12%)	16/41 (39%)

Gene alterations frequency showed that *NRAS* exon 1 mutations were present only in patient with 5 year survival or more, i.e. 1 of 29 (3%), while exon 2 mutations were present in both groups, i.e. 2 of 29 (7%) in patients with longer survival and 3 of 12 (25%) in patients with less than 5 year survival. In no instance were both mutations found in the same sample.

The overall mutation frequency for the *BRAF* gene was 16 of 41 (39%), i.e. 8 of 29 (28%) in patients with longer survival and 8 of 12 (67%) in patients with less than 5 year survival.

Two samples, only found in the group of patients that lived less than 5 years, harbored mutations in both genes (2 of 41 i.e. 5%).

Gene expression profiling study

Using Affymetrix HG-U133 A chips, as an initial step a Welch ANOVA test was performed on the 14 635 probe sets, which were expressed in at least 10% of all samples to compare the gene expression profiles in the three genetic variant classes of melanoma tumor samples. 1 098 (p-value 0.05) probe sets were found to be differentially expressed between melanomas with *NRAS* mutations, *BRAF* mutations and melanomas with neither mutation.

Welch *t*-tests with Benjamini and Hochberg multiple testing correction were applied to the 1 098 probe sets differentiating the 3 genetic variant classes of melanoma in order to compare gene expression differences between any pairs of groups of wt, *NRAS* mutation samples and *BRAF* mutation samples. This analysis resulted in the identification of 417, 450 and 533 differentially expressed genes (p-value < 0.05) when comparisons were made between wt vs. *NRAS* mutation samples, wt vs. *BRAF* mutation samples and *BRAF* vs. *NRAS* mutation samples, respectively. Of 341 overlapping genes, 146 common genes were differentially expressed in wt tumors when compared to both *NRAS* and *BRAF* tumors, but no significant difference between *NRAS* and *BRAF* tumors. Altogether, 99 overlapping genes were generated by comparing wt vs. *BRAF* mutations and *NRAS* vs. *BRAF* mutations. These genes were differentially expressed in the tumors harboring *BRAF* mutations compared to both wt and *NRAS* mutations, while there were no differences between wt and *NRAS*-mutated tumors. There were 95 common genes generated by comparing wt vs. *NRAS* mutation and *NRAS* vs. *BRAF* mutations. These genes showed differential expression patterns in the tumors harboring *NRAS* mutations when compared to wt and *BRAF* mutations. Interestingly, one gene was found to be in common in the three comparisons and had an average expression level that was greatest in *BRAF*-mutated tumors, followed by *NRAS*-mutated tumors and lowest in wt tumors. In contrast, there were 175, 204 and 338 genes showing differential expression patterns for wt vs. NRAS mutation samples, wt vs. *BRAF* mutation samples and *BRAF* vs. *NRAS* mutation samples, respectively.

Supervised hierarchical cluster analysis applied to the 62 melanomas using the 1 098 differentially expressed probe sets in the three different genetic variant classes of melanoma with a p-value < 0.05 revealed two discriminate subgroups, representing a *NRAS*/wt cluster and a *BRAF* mutation cluster. These two subgroups further divided into three separate subgroups representing wt cluster, *NRAS* mutation cluster, and a *BRAF* mutation cluster. Eight of the 62 samples were misclassified in the cluster analysis, i.e. one wt sample was misclassified in the *BRAF* cluster, one wt was misclassified in the *NRAS* cluster, three *NRAS* were misclassified within the wt cluster, and three *BRAF* samples were contained in the larger *NRAS*/wt cluster (Figure 1).

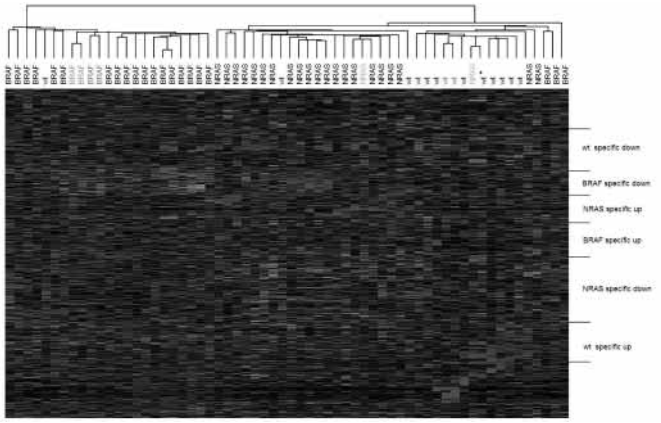

Figure 1. Two dimensional hierarchial clustering of the 1 098 probe sets

In order to identify the genes that distinguish melanomas that are either wt or harbor mutations in *NRAS* or *BRAF*, an arbitrary cut-off value of 1.5-fold (up- or down) mean expression ratios between two classes was applied to the probe sets. This resulted in the identification of a total of 107 differentially-expressed probe sets (representing 97 genes). Of these 107 probe sets, 31 probe sets were differentially-expressed in melanomas that harbored a mutation in *NRAS* (i.e. there were significant differences in both comparisons of wt to *NRAS*-mutated tumors and *NRAS* to *BRAF*-mutated). Furthermore, 39 probe sets were differentially-expressed in melanomas that harbored a mutation in *BRAF* and 37 probe sets were differentially-expressed in melanomas that were wt.

CONCLUSION

Primary melanoma study

Our results indicate that there is no significant correlation between the *NRAS* and *BRAF* mutation frequency and overall survival although mutation frequency was, at least, two times higher in group that lived less than 5 years. Together with this, mutations found in both genes of the same sample (5%) were found in the group of patients that lived less than 5 years, which is higher than previously reported.

Gene expression profiling study

By microarray analysis of 22 215 probe sets, 107 probes sets were found to be differentially expressed between melanoma tissue samples with either *NRAS* or *BRAF* mutations, or neither mutation, suggesting that *NRAS* and *BRAF* mutations may result in different biological effects. This may be expected, since *NRAS* and *BRAF* mutations affect not only overlapping but divergent signaling pathways 14. For patients in whom multiple metastases were available for analysis, 3 out of 5 patients samples clustered correctly, supporting the tenet that in some cases multiple melanoma metastases have common

clonal origins.

The genetic changes responsible for the growth and progression of melanoma remain poorly defined. In addition, the heterogeneity of melanomas continues to thwart efforts to control the disease, and consequently survival of patients with metastatic melanoma continues to be poor. Although activating mutations in *BRAF* and *NRAS* are known to be frequent in melanoma, the consequences of these mutations have not yet been well characterized.

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