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# Coexisting *NRAS* and *BRAF* mutations in primary familial melanomas with specific *CDKN2A* germline alterations

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**To the Editor**

Germline aberrations in the *CDKN2A* gene are observed in some melanoma-prone families and represent high penetrance mutations (Hussussian et al., 1994; Kamb et al., 1994). INK4A (p16) and ARF (p14) are two distinct proteins encoded by the *CDKN2A* locus. Loss of INK4A has been associated with unrestricted cell cycle progression through retinoblastoma (RB) protein inactivation, while loss of ARF has been linked to p53 inactivation with subsequent malfunction in cell cycle regulation, apoptosis and DNA repair (Chin et al., 2006).

The *NRAS* and *BRAF* genes are commonly mutated in sporadic primary cutaneous melanomas, with mutation frequencies between 4–50% (Platz et al., 2008) and 25–80% (Platz et al., 2008), respectively. Codon 61 is the most common position of *NRAS* alterations in melanoma, with frequent glutamine changes to either lysine, Q61K (c. 181C>A), or arginine, Q61R (c.182A>G) (Omholt et al., 2002). Mutations in this residue lock the Ras protein in the GTP-bound state with subsequent continuous activation of its downstream effectors (Platz et al., 2008) through the Ras-Raf-MEK-ERK and Ras-PI3K-Akt pathways. Approximately 90% of reported BRAF mutations occur at residue 600, which is located in the activation domain of this kinase (Thomas, 2006). Current results from melanoma cohorts show that mutations in these genes are almost always mutually exclusive (Edlundh-Rose et al., 2006; Omholt et al., 2003; Platz et al., 2008). Moreover, a high rate of *BRAF* mutation is found also in nevi, suggesting a role in early stages of the neoplastic process (Pollock et al., 2003). Previously, a high frequency of *NRAS* mutations (95%) has been reported in Swedish familial melanoma cases with germline *CDKN2A* alterations (Eskandarpour et al., 2003). The association of *BRAF* somatic mutations with *MC1R* germline variants indicates an influence of constitutive genotype on the preferential acquisition of specific mutations during melanoma development (Landi et al., 2006). In line with this, cooperation between *RAS* and *CDKN2A* has been shown in animal models of melanoma (Chin et al., 1997).

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**Conflict of Interest**
The Authors state no conflict of interest.

Since there is limited information on *NRAS* and *BRAF* mutations in familial melanoma we sought to assess their mutation frequency in melanomas from patients with different *CDKN2A* germline alterations.

The study was performed on formalin-fixed, paraffin-embedded (FFPE) primary familial cutaneous melanomas originating from Brisbane, Australia (16 samples from 15 patients) and Genoa, Italy (3 patients/samples). Clinical and pathological characteristics are shown in Table 1. One patient with a *CDKN2A* L32P mutation had two melanomas originating from the trunk and upper extremity, respectively, while a single melanoma was analyzed from each of the other patients. Eight different germline *CDKN2A* mutations were present in the patients from whom the melanomas were analyzed (Table 2). Laser capture microdissection, DNA extraction, polymerase chain reaction (PCR) amplification, single strand conformation polymorphism (SSCP) and nucleotide sequence analyses of *NRAS* exon 2 and *BRAF* exon 15 were carried out as previously described (Jovanovic et al., 2008; Omholt et al., 2002; Omholt et al., 2003). Each mutation was confirmed by two independent PCR/SSCP analyses followed by sequence analysis performed in both directions. The study was approved by the Ethics Committees of the Queensland Institute of Medical Research, University of Genoa and Karolinska Institutet.

Three (16%) samples had NRAS residue 61 alterations (one Q61R and two Q61K substitutions) while 7 (37%) samples had valine to glutamic acid changes in amino acid 600 of BRAF (V600E; Table 2).

The *NRAS* mutation frequency in this study was lower than we reported previously in melanomas from Swedish families with germline *CDKN2A* mutations (Eskandarpour et al., 2003). The reason for this is unclear. It could possibly be attributed to different origins of studied cohorts (Sweden versus Australia/Italy) and to different *CDKN2A* germline alterations in these two studies (the 112Argdup founder mutation is predominant in Swedish families, which is in contrast to genotypes reported here). However, we cannot exclude that technical factors, possibly related to the fragmented nature of DNA extracted from FFPE samples, may play a role in different rates of mutation detection.

Intriguingly, all 3 tumors with *NRAS* mutations also had *BRAF* V600E mutations. The presence of both *NRAS* and *BRAF* V600E mutations in the same lesions is contrary to the current consensus that such mutations are almost always mutually exclusive in melanomas and other tumor types (Davies et al., 2002; Omholt et al., 2003; Thomas et al., 2007). However, Pollock et al (2003) observed concomitant *NRAS* and *BRAF* V600E mutations in 9% of nevi and suggested this might be due to different clonal nests of cells within these tumors carrying distinct mutations, a possibility that could also explain our findings. Although intriguingly, the joint presence of both *NRAS* and *BRAF* mutations was found only in tumors from patients with *CDKN2A* L32P mutations. In two of these tumors, *NRAS* and *BRAF* mutations (*NRAS/BRAF*: Q61K/V600E and Q61R/V600E) were found in two different DNA extracts while in one case, alterations of both genes (*NRAS/BRAF*: Q61K/V600E) were identified in the same DNA extract. Thus far, it is recognized that a mutation in either *NRAS* or *BRAF* is sufficient for activation of the Ras-Raf-MEK-ERK pathway, with mutant *RAS* having a 50-fold higher activation effect than mutant *BRAF* (Davies et al., 2002). Although we do not have any evidence that the *NRAS* and *BRAF* mutations found in the same DNA extract were coexisting in the same cells, it is possible that the L32P mutation in *CDKN2A* somehow permits cellular tolerance of these dual mutations.

In conclusion, the *NRAS* and *BRAF* mutation rates we observed in familial melanomas were generally lower than most previous reports in sporadic melanoma but equal to those reported for primary melanomas of similar thickness (Goel et al., 2006; Shinozaki et al.,

2004). Samples that harbored INK4A L32P substitutions also had high frequency of coexisting mutations in both *NRAS* and *BRAF*. This suggests that in some instances constitutional *CDKN2A* mutations affect the occurrence of somatic mutations in *NRAS* and *BRAF*, although further work is needed to substantiate this hypothesis.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Table 1

Patient and tumor characteristics

| | Brisbane | Genoa | Total |
|---|---|---|---|
| | N | N | N |
| Number of patients | 15 | 3 | 18 |
| Number of tumors | 16 | 3 | 19 |
| Gender | | | |
| Male | 8 | 2 | 10 |
| Female | 7 | 1 | 8 |
| Median age at diagnosis (range) | 36 (19–56) | 37 (20–41) | 36 (19–56) |
| Site | | | |
| Head and neck | 0 | 1 | 1 |
| Trunk | 6 | 1 | 7 |
| Upper extremities | 6 | 1 | 7 |
| Lower extremities | 4 | 0 | 4 |
| Histology | | | |
| Superficial spreading melanoma | 10 | 2 | 12 |
| Nodular melanoma | 0 | 1 | 1 |
| Unclassifiable melanoma | 6 | 0 | 6 |
| Clark | | | |
| I | 6 | 1 | 7 |
| II | 4 | 1 | 5 |
| III | 2 | 1 | 3 |
| Unknown | 4 | 0 | 4 |
| Mean tumor thickness in mm (SD*) | 0.61 (0.63) | 1.71 | 0.81 (0.92) |
| Ulceration | | | |
| Present | 0 | 0 | 0 |
| Absent | 3 | 3 | 6 |
| Unknown | 13 | 0 | 13 |
| Median survival in months (range) | 372+ (72–942+) | 389+ (257–451+) | 373+ (72–942+) |
| Alive | 12 | 3 | 15 |
| Dead | 3 | 0 | 3 |

*Standard deviation

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**Table 2**

*NRAS* and *BRAF* genotypes in familial melanomas harboring *CDKN2A* germline mutations

| Tumor number | *CDKN2A* mutation | | *NRAS* mutation | | *BRAF* mutation | |
|---|---|---|---|---|---|---|
| | Protein change | Base change | Protein change | Base change | Protein change | Base change |
| 1 | p.A4_P11del | c.9_32del24 | WT‡ | none | p.V600E | c.1799T>A |
| 2 | p.A4_P11dup | c.32_33dup9-32 | WT | none | WT | none |
| 3 | p.A4_P11 dup | c.32_33 dup9-32 | WT | none | WT | none |
| 4 | p.A4_P11 dup | c.32_33 dup9-32 | WT | none | WT | none |
| 5 | p.L16P | c.47T>C | WT | none | WT | none |
| 6 | p.L16P | c.47T>C | WT | none | WT | none |
| 7 | p.R24P | c.71G>C | WT | none | WT | none |
| 8 | p.R24P | c.71G>C | WT | none | p.V600E | c.1799T>A |
| 9 | p.R24P | c.71G>C | WT | none | p.V600E | c.1799T>A |
| 10 | p.L32P | c.95T>C | p.Q61K | c.181C>A | p.V600E | c.1799T>A |
| 11 | p.L32P | c.95T>C | p.Q61R | c.182A>G | p.V600E | c.1799T>A |
| 12 | p.L32P* | c.95T>C | p.Q61K | c.181C>A | p.V600E | c.1799T>A |
| 13 | p.L32P* | c.95T>C | WT | none | WT | none |
| 14 | p.G35A | c.104G>C | WT | none | WT | none |
| 15 | p.G35A | c.104G>C | WT | none | p.V600E | c.1799T>A |
| 16 | p.M53I | c.159G>C | WT | none | WT | none |
| 17 | p.G101W† | c.301G>T | WT | none | WT | none |
| 18 | p.G101W† | c.301G>T | WT | none | WT | none |
| 19 | p.G101W† | c.301G>T | WT | none | WT | none |
| **Total: 19** | | | **3 (16%)** | | **7 (37%)** | |

*Samples originating from the same patient

†Samples originating from Genoa

‡Wild type