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Absence of somatic mutations of NEMO in keratoacanthoma

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To the Editor,

Non-melanoma skin cancer poses a significant clinical problem to organ transplant recipients (OTRs). These patients present with a plethora of different types of skin tumors, in particular squamous cell carcinomas (SCCs) and keratoacanthomas (KAs). KA is considered a benign variant of SCC with the propensity to grow rapidly and regress spontaneously (Karaa et al, 2007). The molecular defects that give rise to SCCs and KAs are poorly understood. However, patients with familial incontinentia pigmenti (IP) develop KAs, particularly subungually (Montes et al, 2004). IP is a rare X-linked disease that is lethal in males and in females leads to a transient inflammatory reaction followed by hyperpigmentation in a distribution pattern that depends on X-chromosome inactivation. IP is caused by mutation or genomic rearrangement of NEMO (NF-κB essential modifier) also referred to as IKBKG or IKKγ. NEMO functions as the regulatory subunit of the IKKα and IKKβ complex and is essential for their kinase activity (Rothwarf et al, 1998). Together, these kinases activate NF-κB in response to a variety of external stimuli including TNFα and IL-1 (Rothwarf et al, 1998). NEMO is required for this response, as embryonic fibroblasts from IP patients cannot activate NF-κB in response to TNFα (Smahi et al, 2000). Under resting conditions, NF-κB subunits are held in the cytoplasm through tethering to IκB proteins. Once stimulated, IKK proteins phosphorylate IκB resulting in its ubiquitination, with subsequent translocation of NF-κB proteins to the nucleus where they activate gene transcription. There is increasing evidence from mouse models that NF-κB signaling is deregulated in skin cancer (van Hogerlinden et al, 1999; Dajee et al, 2003; Park et al, 2007). Silencing of the NF-κB pathway is required for RAS-induced tumorigenesis in a xenograft model (Dajee et al, 2003). Moreover, IκBα is elevated in human SCCs concomitant with the retention of p65, a subunit of NF-κB in the cytoplasm (Dajee et al, 2003). Finally, IKKα deficient mice are more susceptible to chemical induced skin carcinogenesis than wild type mice (Park et al, 2007). Thus, there are several lines of evidence that support the loss of NF-κB activity promotes skin cancer.

In light of the role of the deregulated NF-κB pathway in squamous neoplasia and the increased frequency of KAs in IP patients we assessed the status of NEMO in KA. Sequencing of the NEMO gene is complicated by the presence of a pseudogene (ΔNEMO) located in inverted orientation 22kb away on the X chromosome (Aradhya et al, 2001) (Aradhya et al, 2001). The sequence of ΔNEMO is completely identical with exons 3–10 of NEMO (Aradhya et al, 2001). Thus, female genomic DNA from formalin fixed paraffin embedded tumors (FFPE) contains four identical copies of exons 3–10, two from each gene, which, especially in the presence of DNA from stromal tissue, can make detection of somatic mutations challenging.

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Conflict of interest
The authors declare no conflict of interest.

For this reason, we chose to investigate the presence of somatic mutations in NEMO in KAs from male patients only.

Hematoxylin and eosin sections were reviewed by a dermatopathologist and regions of tumor tissue were identified for dissection. New sections of 30μm thickness were cut from the blocks and the corresponding tumor tissue and, where possible, adjacent normal tissue was collected by microdissection. Genomic DNA was isolated (Bastian et al, 1998) and the concentration determined (Ginzinger et al, 2000). Genomic DNA (5ng) was used to amplify exons 2 to 9 and the coding portion of exon 10 from 21 KAs; 11 from immunocompetent and 10 from OTR patients. The primer sequences are listed in Table 1

We detected one tumor with a non-synonymous mutation resulting in a M407V substitution (Fig. 1a). This mutation has been described previously in IP patients and was thought to be associated with a lethal male phenotype. However, as this mutation was also detected in the normal tissue adjacent to the tumor it is likely to be a germline event in our patient (Fig. 1b). NEMO protein expression was analyzed by immunohistochemistry using a tissue microarray consisting of adjacent normal skin (n=15) and KAs (n=57) (Fig. 2). No significant difference in expression was found between neoplastic keratinocytes and the adjacent histopathologically normal appearing epidermis ($p<0.85$). The tumor carrying the M407V nucleotide change expressed NEMO at comparable levels to the remainder of the cohort. Aradhya et al (Aradhya et al, 2001) detected this variant in only one patient of 357 IP families tested. An additional report also described this variant cosegregating with the disease in an IP family (Smahi et al, 2000). This variant was not found in 60 other unrelated X chromosomes, and thus is likely to be causative rather that represent a polymorphism present in unaffected individuals. From our sequence analyses, we cannot differentiate whether the M407V variation resides within NEMO or ΔNEMO. If the mutation were present in NEMO, our results would indicate that the patient most likely is mosaic, as non-mosaic IP in males is considered lethal.

In the germline, NEMO rearrangements in IP patients predominantly result from a large deletion, affecting exons 4–10. Although, we would expect somatic mutations to mostly consist of point mutations, we cannot fully rule out such large deletions in our cases. Although, it is possible to use long range PCR techniques to detect this rearrangement with high-molecular weight genomic DNA (Bardaro et al, 2003), the degradation of DNA isolated from FFPE tissue precludes the use of this technique. Thus, it is not possible for us to determine whether the mutation occurs in NEMO or ΔNEMO. Nevertheless, our results indicate that somatic mutation of NEMO is an unlikely mechanism relevant in the pathogenesis of KAs. The evidence implicating inactivation of the NF-κB in SCC along with the increased frequency of KAs in IP patients raises the possibility that KA may be caused by somatic mutations within other components of the NF-κB pathway.

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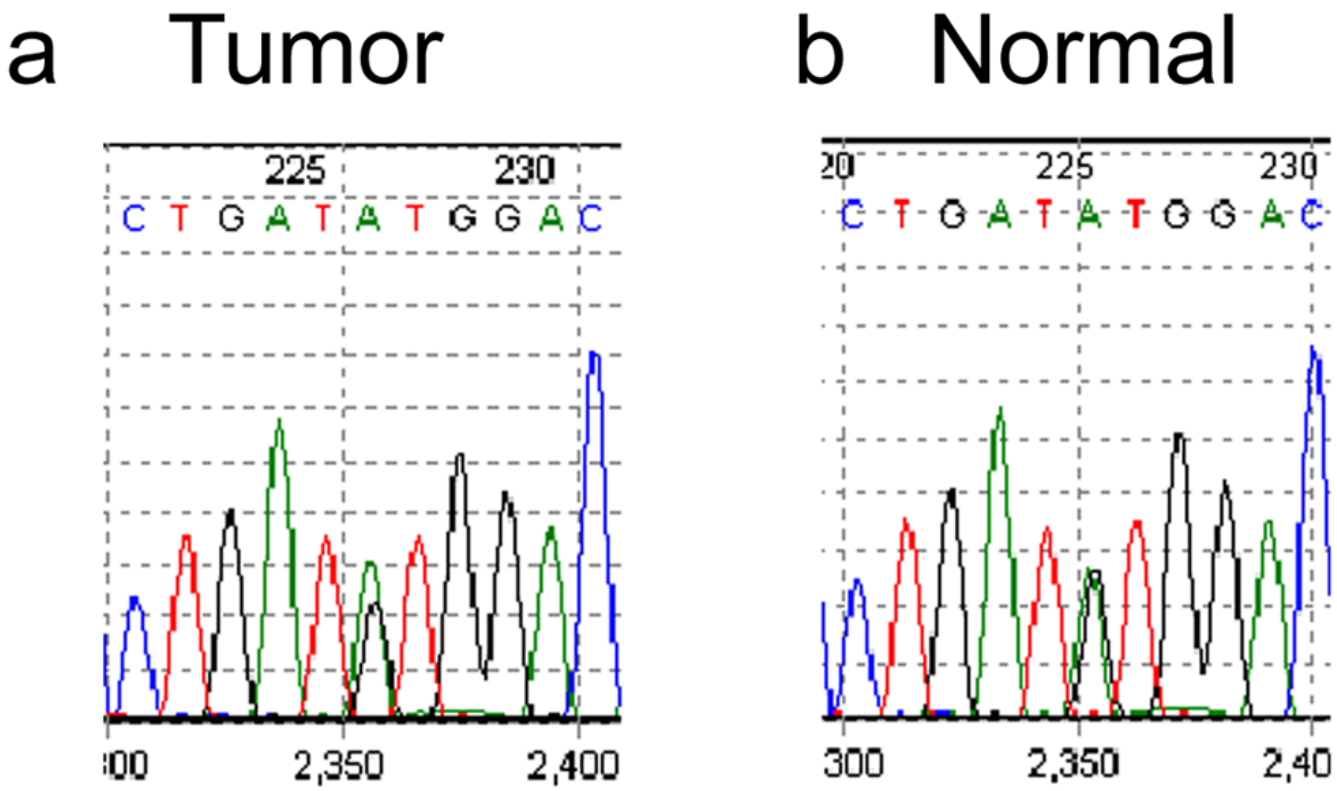

Figure 1. M407V Mutation found in a keratoacanthoma and adjacent normal tissue

The genomic DNA from a keratoacanthoma and adjacent normal tissue was amplified by PCR using primers for exons 2–10 of NEMO. A mutation was found in a keratoacanthoma (a) and the adjacent normal tissue (b).

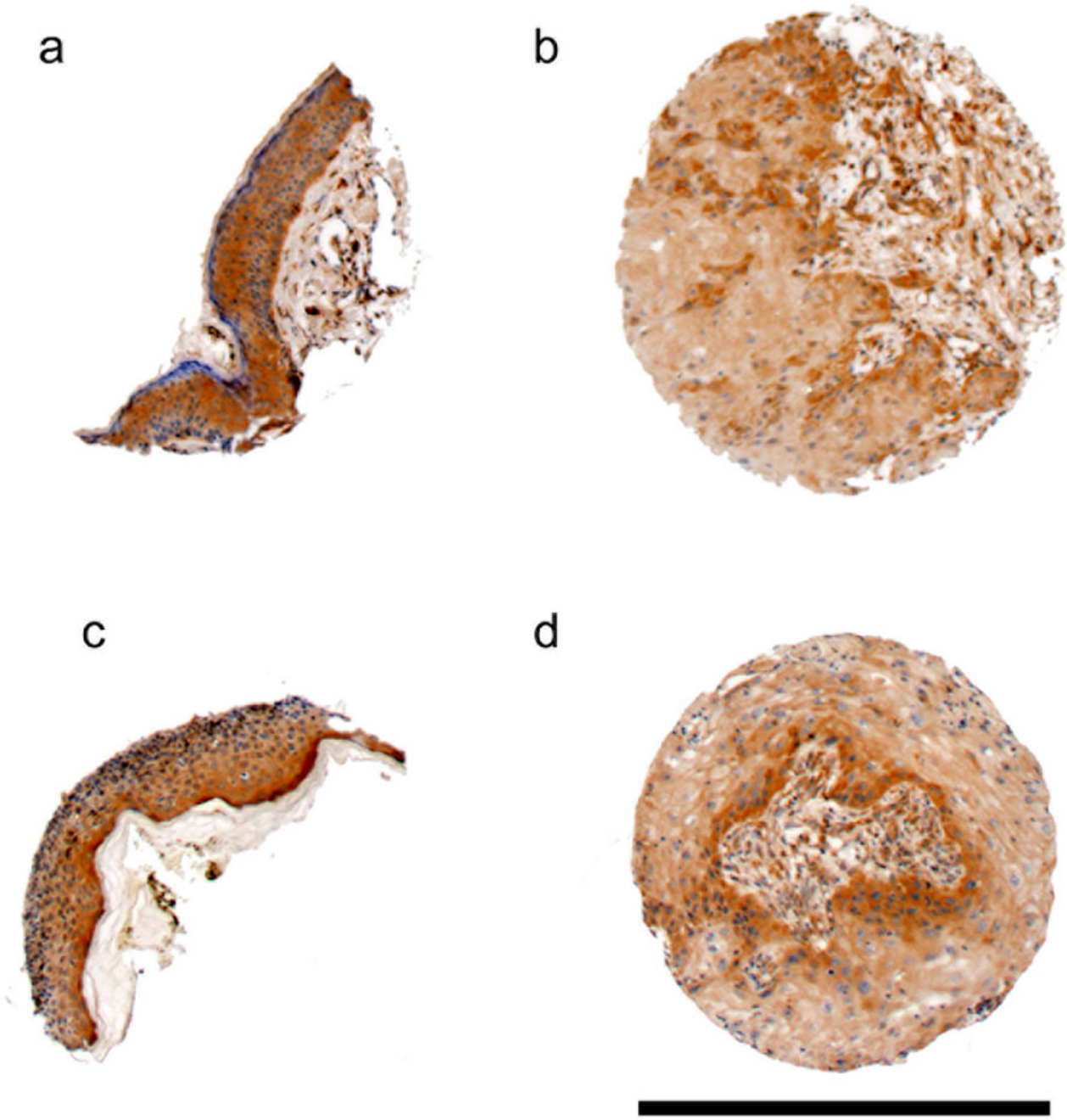

Figure 2. NEMO expression in keratoacanthomas and adjacent normal tissue

Sections were stained with an antibody to NEMO and counterstained with hematoxylin. Representative pictures of adjacent normal tissue (a and c) and keratoacanthomas (b and d) are shown. (scale bar: 1 millimeter)

Table 1

Primers used to amplify exons 2–9 and the coding portion of exon 10 of NEMO

Exon	Forward	Reverse
2	TCTGCTGGGTAAGGATGTGG	GGCTGGATCCCCTGACTCT
3	CCCAGCTCCCCTCCACTGTC	GTGGAACACTGGCGTCAC
4	CAGTGCTGACAGGAAGTGGC	AACCCTGGAAGGGGTCTCCGGAG
5	TCCCTGAGTCTGCTCTTTCC	AGCCTTTCGGCTAAGATCAA
6	AAGGGGGTAGAGTTGGAAGC	AGGCAAGTCTAAGGCAGGTC
7	ACGAGGCTCCGTCAGCTC	ACGCCAAAGAGACTCTCCAG
8	TGCCTGGTGGGTGGCTGGCT	CAGTGTCGCACCCACTGCTCA
9	GCTGCTTTGACACTAGTCCA	CAGAGAGCAACAGGAAGGTC
10	CGGCGGCTCCTGGTCTTACA	GCCACCCAGCCCTTCATCCT