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Letter to the Editor

Expression and function of the apoptosis effector Apaf-1 in melanoma

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Dear Editor,

Melanoma is characterised by its poor response to current therapeutic modalities.[1] Soengas *et al.*[2] showed that Apaf-1, a central component of the intrinsic apoptotic pathway, is often lost in metastatic melanoma, implying that Apaf-1 loss contributes to its remarkable resistance. In our attempts to corroborate their findings in a panel of melanoma lines, we have found no evidence for Apaf-1 downregulation as a major event.

In the paper of Soengas *et al.*,[2] Apaf-1 loss correlates with the inability to execute an apoptotic program upon p53 activation, providing an explanation for the low frequency of p53 mutations in this tumour type. Recently, however, we found that DNA damage-induced cell death in melanoma cells can occur independently of caspase activity[3] or cytochrome *c* release (not shown), and is dependent on an apical serine protease that can side-step the apoptosome to initiate cell death.[3] Moreover, Apaf-1 and caspase-9 deficiency does not facilitate myc-induced lymphomagenesis in mice, arguing against a role for Apaf-1 loss in tumorigenesis.[4] Together, these observations made us re-evaluate the high frequency and relevance of Apaf-1 inactivation in melanoma.

We determined Apaf-1 protein levels in whole-cell lysates of 13 melanoma cell lines and three different melanocyte cultures and found that most lines, except for 607B, demonstrated higher Apaf-1 levels as compared to melanocyte cultures (Figure 1a and not shown). As Apaf-1 exerts its proapoptotic function in the cytoplasm, we also quantified Apaf-1 levels in cytosolic fractions of a selection of these melanoma cell lines. In line with the Apaf-1 levels in whole-cell extracts, cytosolic Apaf-1 levels were higher in most cell lines as compared to melanocytes (Figure 1b). This excludes the possibility that Apaf-1 is inactivated in these lines due to redistribution, similar to what has recently been reported for Burkitt's lymphoma cell lines,[5] and therefore indicates that loss of Apaf-1 expression is rarely found in our panel.

Although our panel contains a relatively high incidence of p53 mutations (5/13), Apaf-1 loss is not correlated to p53 status. As a matter of fact, all melanoma lines that harbour wt-p53 express relatively high levels of Apaf-1 (Figure 1a). The functionality of wt-p53 in these cell lines was confirmed by induction of the p53-inducible gene product $p21^{WAF1}$ after triggering these cells with DNA damage (data not shown). Intriguingly, the only Apaf-1-low cell line in our panel, 607B, harbours mutant p53.[6] These data therefore do not argue for a connection between loss of Apaf-1 and p53 mutation.

We next set out to study the effect of Apaf-1 levels on caspase activation. As caspase-9 is activated by induced proximity[7] and its cleavage is dispensable for its activity, it is not a good measure for enzymatic activity. We therefore measured cleavage of its substrate LEHD-afc in several etoposide-treated melanoma lines (Figure 1c, left panel). This analysis indicated that there is no correlation between caspase-9 activity and Apaf-1 expression (Figure 1a,c). For instance, cell line 607B expresses little Apaf-1 and displayed substantial LEHD-peptide cleavage, while 634 showed clear Apaf-1 expression and no LEHD-peptide cleavage. Besides caspase-9, which is directly regulated by Apaf-1, we also analysed effector caspase activation (DEVDase activity) that is likely to be more relevant for cell death. Also, these assays revealed no correlation between the levels of Apaf-1 and the caspase activity induced by etoposide treatment (Figure 1c, right panel). These results suggest that even though Apaf-1 may be downregulated in some melanoma lines, functional implications for such an event are not clear-cut. An attractive hypothesis that explains these observations is that an alternative, apoptosome-independent mechanism of initiating caspase activation plays an important role in melanoma.

In conclusion, our data confirm that Apaf-1 levels vary in melanoma, but suggest that loss of expression may be less frequent. In this light, it is interesting to note that the Apaf-1 locus has recently been reassessed and shown to be located >0.3 Mbp centromerically on chromosome 12q. Several of the samples that were considered to be Apaf-1 LOH by Soengas *et al.*[8] can therefore no longer be regarded as such when adhering to the rules set by Fujimoto. Although the discrepancy between Soengas' and our data can possibly be explained by the type of melanoma or the treatment regimen given to the patients prior to isolation, we believe it is crucial to realise that loss of Apaf-1 does not necessarily lead to attenuation of caspase activation or of death. It is therefore of importance to nuance the concept that Apaf-1 downregulation is the key event that determines the resistance of melanoma to current treatment modalities.

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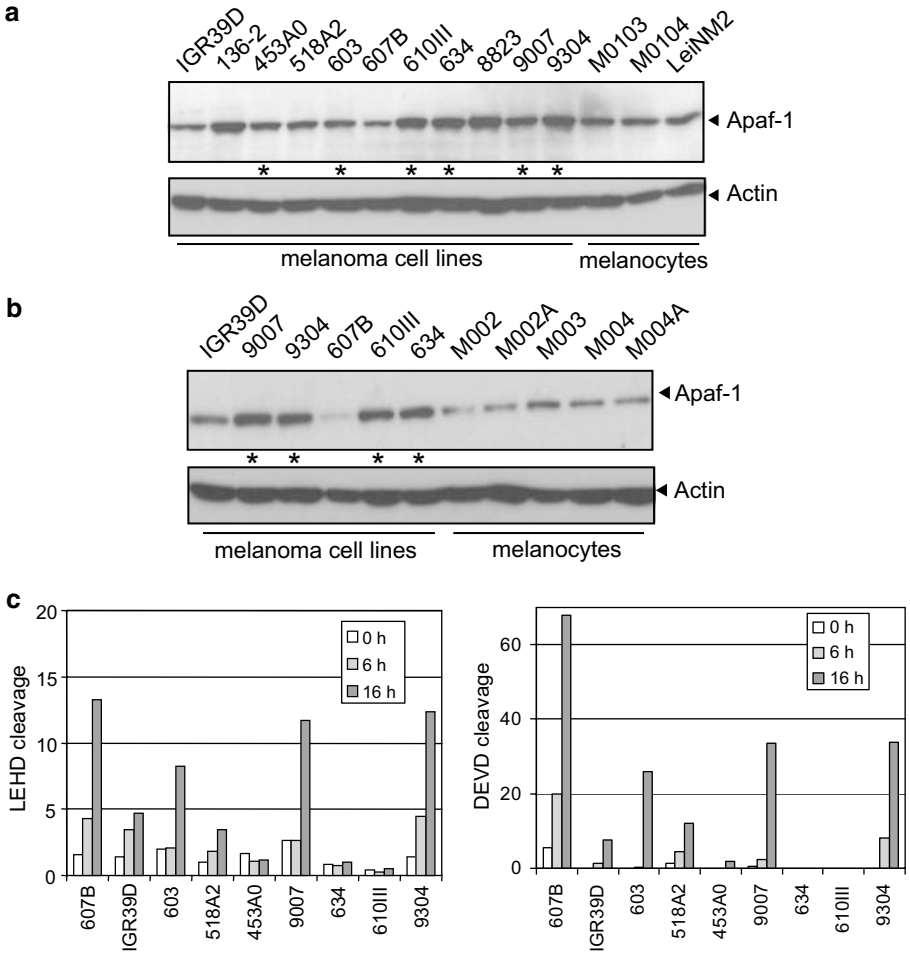

Figure 1 (**a**) Western blot analysis of Apaf-1 of whole-cell lysates[9] of melanoma cell lines and cultured melanocytes[10] derived from normal skin biopsies using a mouse monoclonal antibody (gift of Maria Soengas/Yuri Lazebnik). Asterisks indicate wild type, functional, p53 lines, as analysed using $p21^{waf1}$ upregulation (not shown). These Apaf-1 expression results could exactly be reproduced using a rabbit polyclonal antibody recognising Apaf-1 (gift of X Wang, not shown). Two additional cell lines, which were found to express moderate levels of Apaf-1 and are wt-p53, are not included in this blot. Incubation of the blots with a mouse monoclonal antibody against actin (Amersham, Buckinghamshire, UK) served as a loading control. (**b**) Apaf-1 detected by Western blotting of cytosolic fractions[9] of melanoma cell lines and cultured melanocytes derived from normal skin (M002, M003 and M004) or from the dermal part of a nevus (M002A and M004A) from three different donors (**c**) Caspase-9 activity (left panel) and caspase-3 activity (right panel) in cytosolic fractions of melanoma cell lines treated with 10 μg/ml etoposide (VP16, Bristol-Myers Squibb, New York, NY, USA) for the indicated time periods (h). Cytosolic fractions were prepared by incubation of the cells in swelling buffer, followed by lysis in digitonin-containing buffer.[11] Caspase-9 activity in the cytosolic fractions was assayed in 100 mM Hepes, pH 7.25, 10% sucrose, 0.1% NP-40, 10 mM DTT, by measuring the release of 7-amino-4-trifluoromethyl-coumarin (afc) from LEHD-afc peptides (Alexis, Montreal, Canada). Caspase-3 activity was assayed by measuring cleavage of DEVD-afc peptides (Alexis).[9] The resulting data were sorted according to the estimated relative levels of Apaf-1 protein in the cell lines

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