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# Genetic aberrations and their functional consequences in the multi-step process of skin carcinogenesis

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Non-melanoma skin cancer (NMSC), including the benign and spontaneously regressing keratoacanthoma (KA) and the malignant squamous cell carcinoma (SCC) are frequent human tumors. Their number is still increasing worldwide due to extensive sun exposure. Nevertheless, only little is known about genetic changes and the functional consequences contributing to their formation.

Sun-induced mutation of the TP53 tumor suppressor gene is considered to be an early event in skin carcinogenesis contributing to genome destabilization and thus to tumor formation. We confirm the initiating role of TP53 mutation in skin cancer by immunological detection of aberrant p53 protein being detectable already in single cells in normal human epidermis. The number of mutant cell clones strongly increased in the pre-malignant actinic keratoses and the malignant SCCs, showing aberrant protein in 60 % and 57 % of examined tumors, respectively. In the benign KAs, on the other hand, only 23 % of the tumors show p53 immunostaining. This lower number was due to the absence of p53 staining in 8 KAs derived from the same patient (multiple KAs) suggesting that in those UV-dependent initiation is less crucial.

Another important step in tumor development is up-regulation of telomerase, an enzyme essential for unrestricted growth. So far it is, however, not known at which stage in tumor development telomerase up-regulation occurs. We here show that the catalytic subunit of telomerase, hTERT (human Telomerase Reverse Transcriptase), is detectable by antibody staining in all examined KAs and SCCs suggesting that telomerase up-regulation is not only an important but obviously early step in skin carcinogenesis.

One important aspect of muational inactivation of p53 is that it contributes to tumor development by allowing further genetic changes to occur, i.e. loss of parts of chromosomes causing inactivation of tumor suppressor genes and/or gain of chromosomal regions causing activation of potential oncogenes. We identified such chromosomal changes in KA and SCC by comparative genomic hybridization and demonstrate a very specific aberration profiles for both KAs and SCCs. Unexpectedly, very similar aberrations are involved in both tumor types (e.g. loss of 3p and gain 3q and 11q ), however, in KAs these mostly occur individually in the different tumors while SCCs characteristically show several of these aberrations simultaneously. In addition, SCCs exhibit unique aberrations such as gains of 7q, 8q, 9q, 17q, 20q and loss of 4q and 9p. From this we have to consider that loss of 3p, and gain of 3q and 11q+ are early-stage aberrations (present already in the benign KAs) and it is likely that it is because of the only limited number of aberrations found in KA that these tumors are still able to regress. The accumulation of aberrations of SCCs, causing their highly complex karyotype, on the other hand, seems to be a necessary requisite for malignant progression.

As an example of an “early” and as yet largely undefined aberration we focused on the gain of chromosome 11q13. Interestingly, it was not only a recurrent aberration in KAs and SCCs, but in 4 KAs also the only aberration detectable. By fluorescence *in situ* hybridization we show that the locus of cyclinD1, a candidate oncogene of the 11q13 region, fostering the

G1/ S phase transition of the cell cycle, is even amplified in tumors that do not show 11q gain. Even more so, the cyclinD1 protein is over-expressed in a high number of KAs and SCCs indicating that cyclin D1 up-regulation is not only a frequent but most importantly an early event in the development of NMSC.

To unravel its functional consequence, cyclinD1 was over-expressed in HaCaT skin keratinocytes and the cells were investigated for their *in vitro* and *in vivo* growth and differentiation properties. Interestingly, the transfected cells only gained little growth advantage in conventional (2D) and organotypic (3D) co-cultures (OTCs). However, while HaCaT cells formed a well stratified and orderly differentiated epidermis-like epithelium in OTCs, the transfected cells showed deregulation of tissue architecture with an altered localization of proliferation and impaired differentiation. One clone, that additionally up-regulated cdk4 and p21 even lacked terminal differentiation and formed tumors in two injection sites with a growth pattern resembling that of human KAs. This suggests that cyclinD1 by itself is not sufficient to induce tumorigenicity but it clearly exceeds its known role on proliferation by disrupting tissue organization and thereby allowing abnormal growth. Furthermore, transplantation studies onto the back of immune deficient mice argue for an additional role of cyclin D1 in inflammation.

Another potential oncogene co-amplified with cyclinD1 in the 11q13 amplicon - the myeloma overexpressed gene (Myeov) – could be excluded as a candidate because over-expression studies highlighted its apoptotic function. Thus, our results identified 11q13 gain/cyclinD1 over-expression as an important early step in the formation of NMSC and point to its very specific role in tissue deregulation.

Contrary to cyclinD1, the cell cycle inhibitor $p16^{INK4a}$ (located on 9p21) is often turned off in order to obtain tumor growth. Unexpectedly however, we find high protein levels of $16^{INK4a}$ in 24 of 34 KAs. Since $p16^{INK4a}$ contributes to growth control through inhibition of the cyclinD1/ cyclin dependent kinase 4/6 complex, its up-regulation in KAs may counterbalance the growth promoting function of cyclinD1 and thereby allow for tumor regression. Quite differently, the majority of malignant SCCs (25 /31) lack $p16^{INK4a}$ expression. Since this frequently correlated with loss of 9p21, we assume that the negative immunostaining in SCCs is indeed due to lack of $p16^{INK4a}$ expression. Thus, loss of $p16^{INK4a}$ / 9p21 and with that loss of cell cycle control is likely to be a crucial step from benign to malignant skin tumor progression. Interestingly, a minor fraction, the poorly differentiated SCCs showed $p16^{INK4a}$ staining. The reason for this abnormal behavior remains, however, elusive.

Similar important as cell cycle deregulation is the onset of tumor angiogenesis. Thrombospondin-1 (TSP-1) is supposed to act as an endogenous angiogenesis inhibitor by preventing tumor vascularization and concomitantly tumor cell invasion (malignant growth). In the studies presented here, TSP-1 was detectable in most KAs with the protein being expressed by the tumor cells. In the SCCs, on the other hand, TSP-1 was absent or expressed in the tumor stroma. As also suggested from the literature, this may indicate that TSP-1 is only able to suppress malignant growth when expressed by the tumor itself.

An opponent of TSP-1 is the matrix metalloproteinase MMP-13, a degradative collagenase known to enhance tumor-induced angiogenesis and thereby to add to tumor invasion. We show that expression of this protease is mostly absent in the KAs but present in the SCCs, where staining is particularly prominent at the invading tumor front. Although not firmly proven, this reverse expression of TSP-1 and MMP13 is likely to be of particular significance for malignant conversion and thus an important step in the later stage of skin carcinogenesis.

The results of all these studies now allow to propose a model of skin cancer progression with mutational inactivation of p53, overexpression of cyclinD1 (gain of 11q13) and telomerase up-regulation as early events forcing benign tumor growth. Loss of $p16^{INK4a}$, loss of TSP-1 and upregulation of MMP13, on the other hand are required for the transition from benign to malignant growth leading to cell cycle deregulation and the angiogenic switch

essential for tumor cell invasion. Furthermore, due to this extensive analysis, these data now provide compelling evidence that KAs are not a different tumor entity as frequently suggested but represent a true pre-stage of SCCs which due to lack of the right combination of alterations are still sensitive to environmental control. Since we found a few KAs that closely resembled SCC in their combination of characteristics, it is in addition tempting to suggest that the aberration profile analysed here, has important diagnostic implications in distinguishing benign KAs from malignant SCCs, a fact crucial for the treatment and follow-up of the tumor patients.