mTOR Signalling Pathway-protein Expression in Post-transplant Cutaneous Squamous-cell Carcinomas Before and After Conversion to mTOR-inhibitors

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Abstract. *Background/Aim:* Inhibitors of the mammalian target of rapamycin (mTORis) exert immunosuppressive and antitumor effects and are used in organ-transplant recipients (OTR) as immunosuppressants able to reduce skin tumor burden. This study investigated the effects of mTORis on the expression of mTOR pathway proteins in cutaneous squamous-cell carcinomas (SCC) developing in OTR, before and after switching to mTORis. *Materials and Methods:* An immunohistochemical study was performed on 23 SCC sections excised from OTR with post-transplant SCC, before or after switch to mTORis, with antibodies against pAkt, pmTOR and PI3K. *Results:* pmTOR expression was found in 8/12 SCC pre-switch, and in 8/11 SCC post-switch, to mTORis. All (but 2) SCC expressed PI3K, and all SCCs expressed pAkt. *Conclusion:* mTORis do not significantly change the immunohistochemical expression of molecules upstream of the mTOR inhibition (pmTOR, PI3K, pAkt), in cutaneous SCC.

The PI3K/Akt/mTOR signalling pathway plays a central role in several cell functions, such as growth, proliferation, survival and motility (1). The mammalian target of rapamycin (mTOR), a 289-kDa serine/threonine protein kinase belonging to the family of phosphatidylinositol-3 kinase (PI3K)-related kinases, is the hub of this signalling pathway, acting both as a downstream effector and upstream regulator, and functions in two complexes, mTOR complex 1 and 2 (mTORC1, mTORC2) (2-4). PI3K, a membrane-associated kinase, is the

key molecule upstream of mTOR. Activation of PI3K recruits Akt to the cell membrane, which regulates cell metabolism and mTOR activity (5). Akt is a positive regulator of mTORC1 that phosphorylates and thereby inhibits the heterodimeric tumour-suppressor complex-1 (TSC1/hamartin) and TSC2/tuberlin by suppressing its inhibitory effect on mTORC1 (2). mTOR inhibitors (mTORis), including rapamycin/ sirolimus, temsirolimus, everolimus, and ridaforolimus, form an inhibitory complex which binds a region of mTORC1, thereby inhibiting its activity. In addition to its effects on tumour cells, rapamycin also has antiangiogenic activity (6, 7), which contributes to its clinical antitumor activity. mTORis are used for the treatment of several types of human cancers, either alone or in combination with other chemotherapeutic agents (8-11). As mTORis have also immunosuppressive properties, they are successfully used in organ transplant recipients (OTR) in order to both prevent allograft rejection and to reduce the rate of skin carcinogenesis (12). Indeed, OTR frequently develop squamous-cell carcinomas (SCC) of the skin as a result of chronic immunosuppression (13). It has been shown that conversion of OTRs from calcineurin-inhibitor-based immunosuppression to mTORis reduces the rate of new cutaneous SCC development (14-18), especially when the switch is introduced early in the process of cutaneous carcinogenesis, *i.e.* soon after the development of the first skin SCC (18).

The effect of mTORis on molecules involved in the PI3K/Akt/mTOR pathway has been investigated in some studies using either cell lines or human tumours, but the results have been controversial. Indeed, some studies have found an up-regulation of the molecules upstream of the mTOR inhibition (19-22), whereas other studies (23-25) found opposite results. No relevant study has so far been performed in cutaneous tumours, even though the expression of molecules involved in the mTOR signalling pathway (such as pmTOR) has been previously reported (26, 27). The

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Key Words: mTOR signaling, pmTOR, PI3K, pAkt, squamous-cell carcinoma, skin, organ transplant recipients, immunohistochemistry.

Table I. Antibodies used in the study.

Antibody	Clone	Provider
pmTOR (Ser 2448)	49F9, code 2976	Cell Signaling Technology Inc. (Danvers, MA, USA)
PI3-kinase, p85a	B-9, code SC-1637	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
p-Akt (Ser 473)	D9E, code 4060	Cell Signaling Technology Inc. (Danvers, MA, USA)

aim of the present study was to investigate the effects of mTORis on the expression of proteins involved in the PI3K/Akt/mTOR pathway in cutaneous SCC developing in OTR after switching the patients to mTORis-based immunosuppression.

Materials and Methods

An immunohistochemical study was performed on formalin-fixed, paraffin-embedded specimens of 23 cutaneous SCC retrieved from the files of the Pathology Department, Ed. Herriot Hospital Group, Lyon, France. These tumors had been excised from 10 OTR who had developed post-transplant SCC, and who had been switched to mTORis in order to prevent new carcinoma development, as detailed before (18). The diagnosis of SCC had been confirmed microscopically. Among the 23 cases of SCC studied, 12 (52%) had developed before, and 11 (48%) had developed after, switching to mTORis. The majority of SCC (16/23, 70%) were well-differentiated, 5 were of moderate differentiation and 2 were of low differentiation. 4- μ m thick sections from each SCC were immunostained with antibodies to pAkt, pmTOR and PI3K (Table I). Immunohistochemistry was performed by using a standard streptavidin-biotin-immunoperoxidase technique with diaminobenzidine as chromogen and Mayer's hematoxylin as counterstain, as previously described (27). The SCC were considered positive if at least 10% of tumour cells expressed the molecules examined. Statistical comparison was made with the chi-square test, using the software package SPSS vs. 21. A *p*-value of 0.05 or lower was considered significant.

Results

The expression of pmTOR was cytoplasmic, with a pericellular/membranous accentuation in many cases (Figure 1A). Positivity for pmTOR was found in 8/12 (67%) SCC that had developed before, and in 8/11 (73%) SCC that had developed after, switch to mTORis, this difference being not statistically significant (*p*>0.05). Eleven out of 16 (69%) pmTOR-positive SCC were well-differentiated and 5 (31%) were poorly or moderately differentiated. Five out of 7 (71%) pmTOR-negative SCC were well-differentiated. On the whole, pmTOR expression did not show an obvious correlation with tumor differentiation. pAkt immunoreactivity was observed both in the cytoplasm and nucleus (Figure 1B), and PI3K expression was observed in the cell cytoplasm. All SCC cases in both groups (pre- and post-switch) were positive for pAkt,

and all (but two - one in each group) were positive for PI3K. No statistically significant differences were found in pAkt and PI3K expression between pre- and post-switch SCC cases (*p*>0.05).

Discussion

mTORis are approved for the treatment of several types of human cancers, either alone or in combination with other chemotherapeutic agents (8-10). Everolimus is approved for the treatment of renal cell carcinoma, neuroendocrine pancreatic tumours, hormone receptor-positive, HER2-negative breast cancer and giant cell astrocytoma. However, mTORis are not consistently efficient in tumours with PI3K activation such as metastatic breast cancer (28, 29), suggesting that mTORis are efficacious in some, but not all, tumours.

The effect of mTORis on molecules involved in the PI3K/Akt/mTOR pathway has been investigated in some studies using either cell lines or human tumours, but the results have been somewhat controversial. In one study (19) it was found that rapamycin increased the Akt kinase activity in PI3K-hyperactivated tumour cell lines and colon and breast cancers *in vivo*. Everolimus was reported to increase Akt activity in some, but not all, cell lines from triple-negative breast carcinomas (20). Tabernero *et al.* (21) found that mTORis upregulated pAkt in 50% of human advanced solid tumors. Shi *et al.* (22) observed that mTORis resulted in enhanced Akt activity, phosphorylation and PI3K activity in a myeloma model. Conversely, Li *et al.* (23) found that mTORis reduced Akt phosphorylation in several cancer cell lines. More recently, it was reported that mTORis decreased the expression of Akt and pmTOR in head and neck cancer cell lines, whereas temsirolimus decreased the activity of pAkt (24). A study using Enzyme-Linked Immunosorbent Assay found that everolimus significantly reduced mTOR expression in head and neck SCC (25). The lack of relevant data in cutaneous carcinomas prompted us to comparatively study the expression of these molecules in SCC developing in the absence of, or under, mTORis. We found that the immunohistochemical expression of molecules involved in the mTOR signaling pathway (pmTOR, PI3K and pAkt) did not differ significantly between SCC developed without or

Figure 1. Expression of pmTOR (A) and pAkt (B) in a cutaneous squamous-cell carcinoma (immunoperoxidase revealed with diaminobenzidine, counterstained with Mayer's haematoxylin, original magnification $\times 400$).

under mTORis-based immunosuppression. Considering that conversion of OTRs to mTORis-based immunosuppression decreases the rate of new SCC development (16), these results suggest that the tumor-preventing effect of mTORis is not necessarily reflected by changes in protein expression, at least at the immunohistochemical level. This is in keeping with the results of a previous study (21), which showed, using immunohistochemistry, that mTORis-induced pAkt up-regulation in human advanced solid tumors was not attributable to changes in protein expression. On the other hand, it is likely that even under mTORis-based immunosuppression, the pmTOR pathway remains, at least partly, active; this is consistent with the fact that mTORis does not totally suppress new SCC development, and also with the possibility that mTOR pathway inhibition could result in activation of other pathways involved in carcinogenesis, such as induction of receptor tyrosine kinase (30), suggesting the need for combination treatments targeting several pathways for a more efficient tumour prevention and management.

In conclusion, our results suggest that mTORis do not significantly alter the expression of molecules upstream of the mTOR inhibition, including pmTOR, PI3K and pAkt, in cutaneous SCC, even though mTORis are clinically effective in reducing the rate of SCC development in OTR receiving mTORis-based immunosuppression. Further studies, namely on molecules downstream of the mTOR inhibition, cellular feedback systems or parallel signalling pathways, will hopefully help unravel the molecular mechanisms whereby mTORis reduce skin carcinogenesis in OTR.

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Received April 7, 2018

Revised April 29, 2018

Accepted May 2, 2018