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Chromosomal Aberrations in UVB-induced Tumors of Immunosuppressed Mice

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Abstract

In immunocompromised individuals, such as organ transplant recipients, the risk of cutaneous squamous cell carcinoma (SCC) is increased 60-250 fold, and there is an increased likelihood to develop aggressive, metastatic SCC. An understanding of the genes involved in SCC tumorigenesis is critical to prevent SCC-associated morbidity and mortality. Mouse models show that different immunosuppressive drugs lead to SCCs varying in size, number, and malignant potential. In this study we utilized mouse models that mimic adult transplant recipients to study the effect of immunosuppressive drugs and UV light on SCC development. UV-induced tumors from six treatment groups, control, tacrolimus (Tac), rapamycin (Rap), cyclosporin (CsA), mycophenolate mofetil (MMF), and Rap plus CsA, were evaluated by array comparative genomic hybridization. Mouse SCCs appear to show similar genomic aberrations as those reported in human SCCs and offer the ability to identify genomic changes associated with specific and combinatorial effects of drugs. Fewer aberrations were seen in tumors of mice treated with MMF or Rap. Tumors from Tac treated animals showed the highest number of changes. Calcineurin inhibitors (Tac and CsA) did not cluster together by their genomic aberrations, indicating their contribution to UV mediated carcinogenesis may be through different pathways. The combination treatment (Rap plus CsA) did not cluster with either treatment individually, suggesting it may influence SCC tumorigenesis via a different mechanism. Future studies will identify specific genes mapping to regions of aberration that are different between treatment groups to identify target pathways that may be affected by these drugs.

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Introduction

Post-transplant malignancy is a significant cost of long-term immunosuppressive therapy. There are currently 140,000 organ transplant recipients (OTR) alive in the United States (based on Organ Procurement and Transplantation Network data as of June 2008). Improvements in immunosuppressive regimens, treatment of infectious diseases, and human leukocyte antigen matching have helped increase patient survival. A vast majority of OTR are kept on various levels and combinations of immunosuppressive drugs for their lifetime to maintain function of a transplanted allograft without immunologic rejection.

Nearly all immunosuppressive drugs, whether for treatment of inflammatory diseases or to prevent transplant rejection, are associated with high rates of malignancy (Gutierrez-Dalmau et al., 2007). With increasing survival rates, secondary conditions become a concern for OTR (Berg et al., 2002; Euvrard et al., 2006). Transplant recipients are 27% more likely to develop a solid malignancy after 10 years of immunosuppressive therapy, 60% more likely after 20 years, and 82% more likely after 20 years (Veness et al., 1999). Approximately 90% of all post-transplant malignancies are either squamous cell or basal cell carcinomas. Increased incidence, younger age of onset, higher incidence of multiple tumors, and increased aggressiveness characterize SCC in OTR (Berg et al., 2002). While SCC is a manageable problem for most transplant recipients, it can cause substantial morbidity and even mortality (Berg et al., 2002). Additional risk factors for SCC include UV light, soot, arsenic ingestion, and burns (Boukamp, 2005). Viruses, such as human papilloma virus (HPV) including serotypes HPV-8 and HPV-20, have also been suggested to play a causal role in SCC (Hengge, 2008; Struijk et al., 2008). Very little is known about the genetics and cellular pathways of cutaneous SCC. In OTR, it is not known if the increase of skin cancer is due to suppression of immunosurveillance by the anti-rejection drugs, by direct effect of the drugs on tumor progression, or a combination of these.

The three main types of non-steroidal immunosuppressant drugs used for anti-rejection treatment in OTR are calcineurin inhibitors (CNIs), mammalian target of rapamycin (mTOR) inhibitors, and mycophenolate mofetil (MMF). The two clinically available CNIs are cyclosporine (CsA) and tacrolimus (Tac). CNIs have substantially decreased the risk of acute rejection and improved short term outcomes; however, these drugs have been associated with increased malignancy rates including skin tumors and lymphoma (Gutierrez-Dalmau et al., 2007). In mouse models, both CsA and Tac promote metastasis and angiogenesis and inhibit DNA repair (Yarosh et al., 2005; Duncan et al., 2007). Rapamycin/Sirolimus (Rap) is an mTOR inhibitor that inhibits cell cycle progression and has proven to be a potent immunosuppressive agent for use in OTR (Augustine et al., 2007). Skh-1 mice treated systemically with Rap and chronically exposed to UV irradiation developed more tumors compared to controls. However, the size of the tumors formed in these mice were greatly reduced compared to controls (Duncan et al., 2007). Long-term studies of patients receiving Rap-based immunosuppression show a relatively low incidence of malignancy compared to other immunosuppressants. MMF is an ester prodrug of the immunosuppressant mycophenolic acid. Mycophenolic acid is a selective and reversible non-competitive inhibitor of inosine monophosphate dehydrogenase; an enzyme that is crucial for the proliferation of T and B lymphocytes (Zwerner et al., 2007). In Skh-1 mice treated systemically with MMF and then exposed to UV irradiation the tumor size and number did not significantly differ from UV only treated animals (Duncan et al., 2007). However, large studies of MMF used in various organ transplant patient populations have shown that lymphoproliferative malignancies and non-melanoma skin cancers develop in 0.4-1% and 1.6-4.2% of patients, respectively (Gutierrez-Dalmau et al., 2007). The exact mechanism for immunosuppression induced SCC development is unknown.

From our work with immunosuppressive drugs in mouse models of SCC, we observed key differences in tumor size and multiplicity between drug regimens (Duncan et al., 2007; Wulff et al., 2008). There is also evidence from the literature in human OTR that cancer risk varies between immunosuppressive drug treatments (Gutierrez-Dalmau et al., 2007). Based on these data, we hypothesized that different immunosuppressive drugs may act on different pathways to promote SCC carcinogenesis. The goal of this study was to obtain evidence for this hypothesis using array comparative genomic hybridization (aCGH) to identify different genomic alterations, representing key genes and pathways, between treatment groups. Fifty-one tumors from six different immunosuppressive and control treatment groups were assessed by aCGH to identify genomic changes. We compared our results across groups and compared our data to reported aberrations observed in human SCCs.

Materials and Methods

Mouse Models and Treatment

Adult female Skh-1 hairless mice (Charles River Laboratories, Wilmington, MA) were maintained in an accredited Ohio State University vivarium. Prior to beginning all studies, procedures were approved by the Institutional Laboratory Animal Use and Care Committee. Mice were treated as previously described (Duncan et al., 2007). In brief, mice were exposed dorsally thrice weekly on non-consecutive days for 23 weeks to one minimal erythemic dose of UVB (2240 J/m² as determined by a UVR meter, UVP Inc., Upland, CA) emitted by Phillips FS40UVB lamps (American Ultraviolet Company, Lebanon, IN) that were fitted with TA422 Kodacel filters (Eastman Kodak, Rochester, NY) to ensure the exclusion of UVC light and emission primarily of UVB light (290-320 nm). Drug doses and treatments were as previously described (Duncan et al., 2007). After ten weeks of UV irradiation, mice received, for 13 weeks, CsA, MMF, or phosphate-buffered saline (PBS, control) by intraperitoneal injection (i.p.) once daily at doses of 20 mg/kg/day, while Tac and Rap was given at a dose of 2 mg/kg/day. Drug doses were based on published effective doses in rodents (Blaha et al., 2003). Drug levels were not changed in combination therapy. Mice were euthanized and tissues collected for analysis at the end of week 23.

DNA Extraction

DNA was isolated from sections of tumors or genomic DNA from tail snips by placing frozen tissue into lysis buffer and proteinase K, and incubating at 55°C for 48-72 hours. Residual protein and proteinase K was removed by phenol/chloroform/isoamyl (25:24:1) extraction. Samples were precipitated by adding NaCl and an equal volume of isopropanol followed by a 75% ETOH wash. DNA was resuspended in water, and DNA concentration was measured using spectrometry.

Array CGH and Analysis

DNA from tumors and normal genomic DNA were sent to the UCSF Array Core for their DNA to Data aCGH service (<http://cancer.ucsf.edu/array/index.php>). The UCSF mouse BAC arrays contain 2896 BAC clones spotted in triplicate, with an average spacing between clones of approximately 1 Mb. The arrays used in the study were prepared and hybridized as described previously (Veltman et al., 2003). In brief, reference DNA (isolated from no UV exposure mouse tail) was labeled by random priming using Cy3-dCTP and test DNA (from tumors) labeled by random priming using Cy5-dCTP. Labeled test and reference DNAs were co-hybridized to the arrays. Three single-color intensity images (DAPI, Cy3 and Cy5) were collected from each array using a charge-coupled device camera. The data were normalized using SPOT and SPROC and sent in an excel format with log₂ ratios and genome-wide plots (http://cancer.ucsf.edu/array/protocols/standard_settings.pdf).

Statistical Analysis

Log₂ ratios of BAC clone hybridization intensities from each tumor sample of the six groups were summarized by Box plot and modeled by analysis of variance (ANOVA) method. A cutoff for standard deviation for inclusion in the study was 0.23. To stabilize the variance estimation for BACs, variance smoothing method (Smyth, 2004) was employed. Log₂ ratio differences among treatment groups were tested using estimates from the ANOVA model. The cutoff for exclusion was at least 60% miss values of all BACs. We controlled the mean number of false positives by Gordon's approach (Gordon et al., 2007). Heatmaps were generated for lists of BACs showing differences between the groups for visual evaluation. Hierarchical clustering was performed by using average linkage for both tumor sample and BACs. BACs showing the highest degree of loss or gain for each tumor type were included in the cluster analysis. We used different numbers of BACs for each cluster analysis. They are as follows: All group comparison, 2352 BACs; Tac versus CsA, 130 BACs; Csa versus Rap versus Rap plus CsA, 61 BACs; and MMF versus control, 2352 BACs. We identified the percentage of BACs showing log₂ ratio of greater than 0.3 (gains) and less than -0.3 (losses) for each tumor to determine the percentage of the genome showing aberration. Average frequency gain, loss and overall aberration were measured for each tumor group. Differences of least square means were calculated for each two group comparison. P-values were adjusted for the number of BACs used in the comparisons.

Results

To identify changes in the mouse tumors from different immunosuppressive drug treatment groups and to determine if these changes were similar to those observed in human populations, specific patterns of loss and gain were evaluated in DNA from 51 tumors from controls, Tac, Rap, CsA, MMF and Rap plus CsA treatment groups by aCGH. Array quality was deemed acceptable for further analysis if the standard deviation of clones showing no changes (log₂ ratio=0) was less than 0.23. Seven out of eight control tumors, eight out of ten CsA tumors, five out of six MMF tumors, eight out of eight Rap tumors, eight out of ten Tac tumors, and seven out of nine CsA plus Rap tumors met our criteria of low standard deviation and were included in subsequent analyses. All tumors showed some changes, but the frequency of BAC clones on the arrays that showed changes varied between tumors and between groups (Figure 1).

Comparison Between Human and Mouse aCGH Patterns

From our initial results we hypothesized that chromosomal aberrations seen in human SCC tumors would be similar to the aberrations seen in our mouse models. To determine if this was the case, we compared all published data for human skin tumor chromosomal changes (Table 1) from traditional and array CGH to our mouse data set as a whole. In human SCCs (from OTR and sporadic cases), frequent regions of aberration include 3q gain, 7q gain, 9q gain, 3p loss, 4q loss, and 11q loss (Jin et al., 1999; Popp et al., 2000; Ashton et al., 2003). All of these regions also showed evidence of change in the mouse tumors particularly the regions orthologous to 3p (chromosomes 3, 6, 7, 9 and 14 on mouse; 22-28% gain), 11p (chromosomes 2, 7, and 17 on mouse; 20% loss), and 9q (chromosomes 2 and 4 on mouse; 16-28% gain) (Table 2).

Comparison of aCGH Patterns Between Immunosuppressive Treatment Groups

Based on our data showing differences in tumor multiplicity and size between treatment groups, we hypothesized that SCCs from mice treated with different immunosuppressive regimens would show different aCGH signatures. Because all mice received the same UV exposure, we assumed that any differences between the groups were due to the effect of the immunosuppressive drug treatment. We expected that different immunosuppressive drugs

act through different mechanisms of SCC promotion which would be reflected in different somatic events in tumors. In a comparison between our control treatment mice and all immunosuppressed treatment groups together, we observed a non-statistical trend of more chromosomal aberrations in treated mice than control mice (Fig. 1 and data not shown). We observed the highest frequency of BACs showing losses ($\log_2$ ratio less than -0.3) or gains ($\log_2$ ratio greater than 0.3) in mice treated with Tac (2.9% gain and 2.8% loss) and the least number of BACs showing aberrant copy numbers in mice treated with MMF (0.5% gain and 0.9% loss). After statistical comparisons using difference of least square means we observed borderline significant differences for percentage of total aberration (MMF vs Tac, P-value 0.03; Rap versus Tac, P-value, 0.04); percentage of loss (Rap versus Tac, P-value, 0.04); percentage of gain (CsA versus MMF, P-value 0.05; MMF versus Tac, P-value=0.01). However these differences were not significant following adjustment for the number of BACs in the study.

Histopathology of each of the tumors used for aCGH profiling was not examined. However, we completed histology of 24 tumors from each treatment group at the completion of the study as described (Duncan et al., 2007). In brief, all treatment groups, including UV-only control mice, developed SCCs and papillomas, but of varying numbers. We wanted to rule out the possibility that our interpretation of aCGH patterns observed in the different treatment groups were due to tumor size. One possibility is that tumors of smaller size are more likely to be earlier stages and may therefore show fewer genomic aberrations. We analyzed our data to determine whether the number of BACs showing aberration differed between the small (<2 mm), medium (>2 mm and <10 mm), and large (>10 mm) tumors within a group and did not observe any correlations in size with the number of genomic changes (Fig. 1).

To determine if there were specific gains and losses that could be used to differentiate between each of the treatment groups, we performed an unsupervised cluster analysis of all single treatment groups. We first eliminated BACs from study that did not yield results in 60% of hybridizations. Next, we removed BACs that did not show a high frequency of change in any of the treatment groups. The remaining BACs (n=2352) were used in the unsupervised cluster analysis. Unsupervised analysis did not separate any of the tumor groups with 100% efficiency (data not shown). Tumors clustered into two main groups, a group showing a higher degree of genomic aberration and a group showing less overall genomic aberration. The only treatment group in which at least 50% of the tumors clustered immediately together was Tac. The group showing the least amount of similarity between the tumors was CsA in which none of the eight tumors in the study clustered immediately adjacent to other tumors. These data suggest that there are some similarities in specific genomic aberrations between treatment groups, but also indicate that there are differences in overall genomic stability in SCCs between groups.

Because tumors from MMF treated animals showed the least number of overall genomic changes, we were interested if there were differences in existing genomic changes between MMF and control groups. Unsupervised cluster analysis was not able to distinguish between these two groups, suggesting that the regions of copy number changes in SCCs treated with UV alone or MMF are similar (Fig. 2).

CsA and Tac are both calcineurin inhibitors. From our previous studies, we observed differences in tumor number and size between CsA and Tac treated mice (Duncan et al., 2007). Nonetheless, we expected that tumors from these two groups would show similar patterns of loss and gain since they affect the same immunosuppressive pathway. To test this hypothesis, we completed unsupervised cluster analysis of tumors from CsA and Tac treated mice using 130 BACs shown to have changes in one or both groups. We observed

separation of the CsA and Tac tumors with six of eight Tac tumors clustering together and five of eight tumors from CsA treated animals clustering together (Fig. 3).

In this study, we had tumors from one two-drug combination group, Rap plus CsA. Based on the extremely different pathways that Rap and CsA are predicted to effect, we expected that tumors from the two groups would show quite different patterns of genomic gains and losses. We did not know whether the combination treatment group would show a unique signature or if it would be more likely to cluster with one or the other single treatment group. To test these possibilities, we performed cluster analysis of Rap versus CsA versus combination of Rap plus CsA. The Rap and CsA tumors from the single treatment groups did cluster separately, but to our surprise were more similar to each other (on same major branch) than to the Rap plus CsA combination. In general, the Rap plus CsA combination showed fewer genomic changes when compared to the single treatment groups. The three CsA tumors which clustered within the Rap plus CsA treatment group showed fewer overall changes than the other CsA tumors (Fig. 4).

Complete correlation between copy number changes and the genes involved has not been completed. We queried a handful of genes that have been implicated to play a role in UVB SCC induction or tumorigenesis to determine if they mapped to BACs showing significance differences between treatment groups and controls. These included *Prkab1* (AMP-activated kinase) and *Tnfa*, both of which have been shown to be induced by UV in keratinocytes (Cao et al., 2008; Faurschou et al., 2008a). Tumors from Tac treated animals had gains of *Prkarb1* and *Tnfa* (P-value=0.00000458). Genes in the Tac treatment group showing significant copy number gains include *Ccnd1* (cyclin D1) and *Nfatc2* (NFAT1) and losses include *Bad*, *Rb* and *Fkbp1a*. The BAC containing *Tnfa*, RP23-104F16, also showed gains in CsA/Rap and Rap tumors and the BAC containing *Nfatc2*, RP23-107C9, was gained in the CsA/Rap combination tumors. The BAC containing *Akt*, RP23-440M10, showed gains (P-value=0.04) in Rap treated tumors; it is not known if the tumors were trying to overcome the inhibition of mTOR by Rap treatment. More detailed analysis of these pathways and genes will provide important information as to whether these pathways appear to be important for SCC development in the context of specific immunosuppressive drug treatment.

Discussion

Here, we present the first aCGH data from SCCs developing in the context of specific immunosuppressive drugs. Immunosuppressed populations such as transplant recipients, HIV/AIDS patients, cancer patients, and patients with autoimmune diseases are at a higher risk for developing nonmelanoma skin cancers, especially SCC (Mitsuyasu, 2008). Organ transplant recipients are at the highest risk and demonstrate more aggressive characteristics than skin cancers in non-transplant recipients. This is thought to be due to a combination of sun exposure and the continued administration of immunosuppressive drugs to prevent or treat organ rejection.

It has been difficult to determine which specific immunosuppressive drugs are responsible for the increased skin cancer risk and the general mechanisms underlying the increased risk due to varying drug regimens (doses, combinations and changes over time) after transplant and varying post-transplant sun exposure behavior modification. To alleviate some of the difficulties in using SCCs from OTR, we used the Skh-1 hairless mouse model of skin cancer to study the effects of specific immunosuppression regimens on genomic aberrations in UVB-induced SCC tumors. Skh-1 mice have been used successfully to study UV-induced SCCs with and without immunosuppressive drug treatment (Saul et al., 2005; Duncan et al., 2007; Thomas-Ahner et al., 2007; Tober et al., 2007; Wulff et al., 2008). We were interested

to determine if our mouse models would be useful for the understanding of genomic changes occurring in human SCCs developing in immunosuppressed hosts. Our mouse data show many similarities with the published human SCC aCGH data. If genomic patterns of loss and gain are an estimate of tumor suppressor or oncogene involvement, our data suggest that some of the same tumor suppressor and oncogenes involved in SCC development in humans are also involved in SCC development in our Skh-1 mouse models. This indicates that SCCs from the Skh-1 mice should be an excellent model for studying the relationship of immunosuppressive drug treatment and SCC development. Further use of these models will help in the understanding of the role the different drug types play in human SCC development. By using mouse models one can control for drug treatment and dosage and can avoid the complications of studying these changes in human OTR.

This study was focused on identifying immunosuppressive specific effects on genomic changes in tumors. There are a number of other risk factors for SCC including UV-exposure, HPV, and other carcinogens. *TP53* mutations are known to be an early event in UV-induced carcinogenesis of both mouse (60-65%) and human (90%) SCCs (Benjamin et al., 2008; Verkler et al., 2008). As all of the mice in our study were treated with UVB, we expect that UVB induced changes including *Trp53* mutation status were similar in all treatment groups.

We did not look at the proportion of each cell type present in our tumor samples prior to isolation of DNA. Thus, it is possible that some tumors contained more non-tumor cells such as lymphocytes than others. This could lead to a general “undercalling” of somatic changes in the tumors. We also cannot rule out the possibility that some of the copy number changes that we observed were confined to changes in stromal cells. In general, we observed more genomic instability in our tumors from mice treated with the calcineurin inhibitors (CsA, Tac) than mice treated with control. These data suggest that some of the immunosuppressive drugs may act by targeting pathways that result in genomic instability.

Not all of the immunosuppressive treatments showed more genomic alterations; the least number of genomic aberrations was observed in our MMF group. MMF acts as a reversible, noncompetitive inhibitor of the enzyme inosine monophosphate dehydrogenase (IMPDH). IMPDH is necessary for one mechanism of purine synthesis in most cell types and is required for purine synthesis in lymphocytes. The end result of MMF treatment is decreased DNA production and a subsequent effect on activated B and T lymphocytes. MMF does not intercalate into DNA and induce mutations; therefore it is believed that MMF may have less oncogenic potential (Zwerner et al., 2007). A study involving over 6000 renal transplant patients using MMF showed no evidence of increased risk of developing any malignancy when compared to other immunosuppression regimens. The MMF treated group actually showed a trend toward a decrease incidence in malignancy and a significant increase in time to malignancy (Zwerner et al., 2007). Since fewer genomic changes were observed in tumors from MMF treated mice, tumorigenesis could be driven by UV-induced mutation, methylation, microsatellite instability or specific activation of oncogenic pathways similar to those in the control group.

We examined two calcineurin inhibitors in this study, Tac and CsA, both of which target different pathways. Our data are quite intriguing as they imply that CsA and Tac, although both calcineurin inhibitors, may impact different pathways during carcinogenesis. Calcineurin has been indicated to play a role in the differentiation of keratinocytes (Santini et al., 2001) and melanocyte pigmentation (Lee et al., 2003). Smit et al. (2008) found a higher level of calcineurin activity in the epidermis containing keratinocytes and fibroblasts than the dermis. This indicates that keratinocytes may be direct targets for calcineurin inhibitors. Interestingly, in this study Tac showed stronger immunosuppression than CsA. This may explain why we observed a trend towards more aberrations in our Tac treated

tumors compared to our CsA treated tumors (Smit et al., 2008). In vitro, Tac and CsA form complexes with intra-cytoplasmic immunophilin proteins. CsA binds to cyclophilin A and Tac binds to a 12-kDa protein called tacrolimus binding protein (FKBP12) (Staat et al., 2004; Iwasaki, 2007; Weischer et al., 2007). FKBP12 has been shown to be expressed in keratinocytes and is believed to play a role in their differentiation (Nishio et al., 2000). The binding partner differences may also account for why we see more genomic instability in the Tac treated mice as the Tac and CsA protein complexes may have additional targets. An in vitro study demonstrated that Tac had an inhibitory effect against human liver cancer cells, and it inhibits 12-O-tetradecanoyl phorbol-13-acetate (TPA) induced promotion of skin papilloma formation in *CD-1* mice (Iwasaki, 2007). Other studies have shown that Tac increases the levels of TGF β 1, thereby promoting tumor progression and metastasis (Staat et al., 2004; Iwasaki, 2007). Taken together with the current studies, the differential effect of calcineurin inhibitors Tac and CsA on skin tumor formation needs further investigation.

We also examined the effects of combined Rap plus CsA on SCC development because it is a commonly used immunosuppressive combination therapy for OTR. In general the Rap plus CsA combination showed fewer genomic changes compared to the single CsA treatment group which may be a reflection on the number of pathways being targeted or may be due to the influence of Rap which shows few genomic aberrations overall. Rap is effective when combined with a variety of other immunosuppressive agents. Like Tac, it binds to FKBP12. Rap is a proliferation signal inhibitor that blocks growth factor-induced transduction of signals that mediate cellular division in response to alloantigens by inhibition of mTOR (Augustine et al., 2007; Weischer et al., 2007). Inhibition of mTOR blocks the activation of the p70^{S6} kinase, which leads to cell cycle arrest at the G₁ phase of the cell cycle, which in turn inhibits IL-2 receptor and CD28 dependent signaling pathways. Compared to CNIs, which block the production of cytokines, Rap blocks the ability of the cytokines to act and so prevents growth factor-driven proliferation of both hematopoietic and non-hematopoietic cells. Rap blocks effector functions of CD4⁺ T helper cells and CD8⁺ cytotoxic T cells, the activation of monocytes and the proliferation and differentiation of B cells. Rapamycin is also thought to prevent UVB-induced immunosuppression in skin by inhibiting expression of TNF α through a DNA damage-FRAP-TP53-linked pathway (Al-Daraji et al., 2002; Yarosh et al., 2002). TNF α overrides the G2/M checkpoint in premalignant skin cells and allows for some cells containing unrepaired cyclobutane pyrimidine dimers to enter the cell cycle (Faurischou et al., 2008b). We saw increased copy numbers of TNF α in our Tac, CsA, and Rap treated tumors. Long term studies of patients receiving Rap-based immunosuppression show a relatively low incidence of malignancy compared to that seen with other immunosuppressants. Several experimental models have found that mTOR inhibitors, like Rap, have a negative effect on tumor cells. For example, Rap alone, or in combination with CsA prevents metastatic tumor progression and prolongs the survival of mice inoculated with renal cancer cells but CsA treatment alone increases the number of metastases (Augustine et al., 2007; Weischer et al., 2007). Our data fit the idea that Rap together with CsA affects tumorigenesis differently than when each of the immunosuppressive drugs is used in isolation. It is also interesting that our Rap treated tumors show a significant gain of TNF α compared to control treated tumors. This could be an indication that these tumors are trying to overcome the Rap induced inhibition of TNF α . Future studies will be completed to test this possibility.

As the risk of SCCs is increased 65-250 fold in OTR and much of this risk is thought to be attributable to immunosuppressive drug regimens, an understanding of the genomic changes that occur during SCC following specific immunosuppressive drugs may lead to a better understanding of how to decrease SCC risk in this population. The Skh-1 mouse models appear to show many similarities to changes occurring in human SCCs and offer the ability to identify genomic changes associated with specific and combinatorial effects of drugs.

Using these models in subsequent studies, we expect to advance our knowledge of SCCs in human populations. Future studies will begin to address the specific genes that show different genomic aberrations between the treatment groups to identify target pathways that may be affected by these drugs.

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Figure 1. Percentage of BACs showing gains or losses in each treatment group. A. Percentage of BACs showing loss by treatment. B. Percentage of BACs showing gains by treatment. Small tumors (<2 mm) are shown in red squares; medium tumors (between >2 mm and <10 mm) are shown in blue circles; large tumors (>10 mm) are shown in green triangles; averages for each treatment are represented by the bolded line.

Figure 2.

Copy number aberrations in control and MMF treated tumors. Unsupervised hierarchical cluster analysis using 2352 BACs was performed on seven PBS-treated tumors (control) and five MMF treated tumors. A representation of the cluster plot is shown. Gains are shown in red and losses in green. Both the MMF and control treated tumors show few copy number changes and are not able to be separated by unsupervised cluster analysis, suggesting that many of the changes occurring in these two groups are similar.

Figure 3.

Copy number aberrations in calcineurin treated tumors. Unsupervised hierarchical cluster analysis using 130 BACs was performed on eight CsA-treated tumors and eight Tac treated tumors. Gains are shown in red and losses in green. **A.** The complete cluster analysis is shown with the boxed region indicating the cluster highlighted in B. **B.** A close up view of a portion of the cluster analysis is shown. CsA and Tac segregate on cluster analysis. CsA tumors are underlined in orange and Tac clusters are underlined in blue.

Figure 4.

Copy number aberrations in CsA, Rap, and Rap plus CsA combination treated tumors. Unsupervised hierarchical cluster analysis using 61 BACs was performed on eight CsA, eight Rap, and seven Rap plus CsA combination treated tumors. Gains are shown in red and losses in green. Tumors from the Rap plus CsA combination clusters independently of tumors from CsA and Rap treated animals and show fewer genomic alterations. Using these 61 BACs, tumors from CsA and Rap treated animals cluster near each other, suggesting that they share many genomic changes. Rap plus CsA tumors are indicated by a purple line, CsA tumors by an orange line, and Rap tumors by an aqua line.

TABLE 1
Genetic Imbalances Detected by Karyotype, CGH, and SNP Microarray in Human SCCs

Gains			Losses				
Chr	Karyotype (Jin et al., 1999)	CGH (Popp et al., 2000; Ashton et al., 2003)	SNP Array (Purdie et al., 2007)	Chr	Karyotype (Jin et al., 1999)	CGH (Popp et al., 2000; Ashton et al., 2003)	SNP Array (Purdie et al., 2007)
1q	13.6% (3/22) ^a	21.1% (4/19)	31% (5/16)	2q	13.6% (3/22) ^a	0% (0/19)	50% (8/16)
2p	4.5% (1/22)	10.5% (2/19)	n/a	3p	9.1% (2/22)	63.2% (12/19)	56% (9/16)
3q	9.1% (2/22) ^a	57.9% (11/19) ^a	37% (6/16)	4p	27.3% (6/22) ^a	0% (0/19)	n/a
4p	0% (0/22)	21.1% (4/19)	n/a	4q	27.3% (6/22) ^a	21.1% (4/19) ^a	n/a
4q	0% (0/22)	10.5% (2/19)	n/a	5p	13.6% (3/22) ^a	0% (0/19)	n/a
5p	13.6% (3/22) ^a	31.6% (6/19) ^a	n/a	5q	4.5% (1/22)	31.6% (6/19)	n/a
7p	31.8% (7/22) ^a	31.6% (6/19)	31% (5/16)	8p	36.4% (8/22)	36.8% (7/19)	50% (8/16)
7q	22.7% (5/22)	15.8% (3/19)	n/a	9p	22.7% (5/22) ^a	52.6% (10/19) ^a	81% (13/16)
8q	27.3% (6/22)	42.1% (8/19)	37% (6/16)	9q	4.5% (1/22)	10.5% (2/19)	25% (4/16)
9q	18.2% (4/22)	15.8% (3/19)	25% (4/16)	10p	18.2% (4/22)	10.5% (2/19)	n/a
10q	9.1% (2/22)	15.8% (3/19)	n/a	10q	9.1% (2/22)	15.8% (3/19)	n/a
11p	13.6% (3/22)	5.3% (1/19)	n/a	11p	27.3% (6/22) ^a	15.8% (3/19)	25% (4/16)
11q	13.6% (3/22) ^a	21.1% (4/19) ^a	n/a	11q	13.6% (3/22) ^a	10.5% (2/19)	n/a
14q	4.5% (1/22) ^a	26.3% (5/19)	n/a	13	27.3% (6/22)	15.8% (3/19)	50% (8/16)
16	4.5% (1/22) ^a	10.5% (2/19)	n/a	14	22.7% (5/22) ^a	0% (0/19)	n/a
17q	0% (0/22)	36.8% (7/19) ^a	n/a	15	18.2% (4/22) ^a	10.5% (2/19)	n/a
20	18.2% (4/22)	15.8% (3/19)	n/a	16	13.6% (3/22)	5.3% (1/19)	n/a
21	4.5% (1/22)	26.3% (5/19)	n/a	17p	9.1% (2/22)	47.4% (9/19)	n/a
				17q	9.1% (2/22)	10.5% (2/19)	25% (4/16)
				18p	18.2% (4/22)	36.8% (7/19) ^a	n/a
				18q	27.3% (6/22)	36.8% (7/19) ^a	31% (5/16)
				19q	13.6% (3/22) ^a	0% (0/4)	n/a
				21p	40.9% (9/22)	0% (0/19)	n/a

Gains			Losses				
Chr	Karyotype (Jin et al., 1999)	CGH (Popp et al., 2000; Ashton et al., 2003)	SNP Array (Purdie et al., 2007)	Chr	Karyotype (Jin et al., 1999)	CGH (Popp et al., 2000; Ashton et al., 2003)	SNP Array (Purdie et al., 2007)
				21q	27.3% (6/22)	5.3% (1/19)	n/a
				22	18.2% (4/22)	0% (0/4)	n/a
				X	22.7% (5/22)	10.5% (2/19)	n/a
				Y	31.8% (7/22)	0% (0/4)	n/a

Only changes seen in at least 10% of samples tested are listed

a partial arm;

n/a, not applicable

Chr, Chromosome arm

TABLE 2

Orthologous Human Loci to Mouse aCGH Aberrations

Human Chromosome	Mouse Location ^a	% of tumors gain (n)	% of tumors loss (n)
1p	chr3, chr4	11-29% (34-44)	11-20% (34-44)
1q	chr3, chr1	13-20% (43-45)	13% (45)
2p	chr6, chr11, chr12, chr13	24% (45)	11-13% (36-45)
2q	chr1	11-24% (37-45)	
3p	chr3, chr6, chr7, chr9, chr14	11-17% (45)	22-28% (45)
3q	chr3, chr16	15% (45)	11-20% (44)
4p	chr5	11%(44-45)	11% (45)
4q	chr3, chr4, chr5, chr8	19% (36)	11-30% (40-45)
5q	chr11, chr13	16% (36)	11% (43-45)
6p	chr1, chr13, chr17	11-13% (43-45)	11-35% (42-45)
7q	chr1, chr5, chr6	40% (44)	15-38% (45)
8q	chr4		11-22% (42-45)
9q	chr4, chr2	16-28% (42-45)	
10q	chr19, chr14	11-20% (44-45)	11% (43)
11p	chr17, chr7, chr2	23-35% (42-43)	20% (43-45)
11q	chr4, chr7, chr9	15% (44-45)	11-17%(45)
12p	chr6	13-15% (45)	
12q	chr15	13% (30-44)	
13q	chr14	11% (44)	11% (44)
14q	chr12, chr2	20% (39)	13-16% (43-44)
15q	chr7, chr2		13-22%(44-45)
16p	chr7, chr2	15-37% (44-45)	
17q	chr11	11-16% (37-45)	20% (45)
19p	chr2, chr9, chr17	13% (43-45)	17% (45)
19q	chr7, chr2	13-52% (37-45)	
20q	chr2		12% (39)
22q	chr7, chr12, chr15	16-31% (41-43)	13-17% (45)

^a Only regions showing loss or gain in at least 10% of tumors and in more than one BAC per region are shown.