Squamous Cell Tumors in Mice Heterozygous for a Null Allele of \textit{Atp2a2}, Encoding the SERCA2 Ca\textsuperscript{2+} Pump

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Running title: SERCA2 mutation and squamous cell tumors

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SUMMARY

Mutations in the human ATP2A2 gene, encoding sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase isoform 2 (SERCA2) cause Darier disease, an autosomal dominant skin disease characterized by multiple keratotic papules in the seborheic regions of the body. Mice with a single functional Atp2a2 allele (the mouse homolog of ATP2A2) were shown previously to have reduced levels of SERCA2 in heart and mildly impaired cardiac contractility and relaxation. Here we show that aged heterozygous mutant (Atp2a2\(^{+/-}\)) mice develop squamous cell tumors of the forestomach, esophagus, oral mucosa, tongue, and skin. Squamous cell tumors occurred in 13/14 Atp2a2\(^{+/-}\) mice, but were not observed in age- and sex-matched wild-type controls. Hyperkeratinized squamous cell papillomas and carcinomas of the upper digestive tract were the most frequent finding among Atp2a2\(^{+/-}\) mice, and many animals had multiple tumors. Western blot analyses showed that SERCA2 protein levels were reduced in skin and other affected tissues of heterozygous mice. The development of squamous cell tumors in aged Atp2a2\(^{+/-}\) mice indicates that SERCA2 haploinsufficiency predisposes murine keratinocytes to neoplasia. These findings provide the first direct demonstration that a perturbation of Ca\(^{2+}\) homeostasis or signaling can serve as a primary initiating event in cancer.
INTRODUCTION

Sequestration of Ca\textsuperscript{2+} in intracellular storage organelles, from which it can be released during Ca\textsuperscript{2+} signaling events, is mediated by the SERCA\textsuperscript{1} family of Ca\textsuperscript{2+}-ATPases. SERCA2 (gene locus symbol: \textit{Atp2a2} for mouse, \textit{ATP2A2} for human), the most widely expressed SERCA isoform, has two C-terminal variants (1, 2). SERCA2a is expressed primarily in heart, where it mediates cardiac muscle relaxation and maintains sarcoplasmic reticulum Ca\textsuperscript{2+} stores on a beat-to-beat basis; SERCA2b is expressed in all tissues and is thought to be the major endoplasmic reticulum Ca\textsuperscript{2+} pump (3). Gene targeting studies in mice have shown that at least one functional copy of the \textit{Atp2a2} gene is essential for survival and that the loss of a single allele causes reductions in SERCA2 mRNA and protein in heart (4). Despite some compensation via alterations in the levels and phosphorylation status of phospholamban, which regulates the pump, cardiac muscle contractility and relaxation were impaired and intracellular Ca\textsuperscript{2+} transients in cardiac myocytes were reduced (4, 5).

Darier disease, an autosomal dominant skin disorder in humans (6), has been shown to be due to null mutations in one copy of the \textit{ATP2A2} gene (7), demonstrating that SERCA2 haploinsufficiency in keratinocytes can cause disease. To determine whether SERCA2 haploinsufficiency would lead to disease in mice as they age, we conducted an aging study using wild-type and \textit{Atp2a2}\textsuperscript{+/−} mice. Subsequent analyses revealed that heterozygous mutants have a very high incidence of squamous cell carcinomas and papillomas in keratinized epithelial cells, the same cell type affected in human Darier disease. Although previous studies have provided suggestive evidence that perturbations of intracellular Ca\textsuperscript{2+} homeostasis or signaling may contribute to cancer (8, 9), the data presented in the current study establish the first direct link between altered Ca\textsuperscript{2+} handling and neoplasia.
EXPERIMENTAL PROCEDURES

Mice—The Atp2a2 gene was targeted previously by removing the promoter and first two exons, which eliminated expression of the mutant gene (4). Atp2a2+/− mice of a mixed background (50% 129/Svj, 50% Black Swiss) were crossed to obtain wild-type and Atp2a2+/− mice, and genotypes were determined by PCR analysis of tail DNA as described previously (4).

Aging study and histology—Sex-matched heterozygous and wild-type sibling-pairs were aged and animals exhibiting evidence of morbidity, such as wasting, open sores, or apparent tumors, were euthanized. At necropsy, the skin, nails, and major organs were examined for gross lesions or abnormalities. The mouth and tongue were not examined in the first four Atp2a2+/− mice exhibiting disease symptoms, but were included in the protocol after we became aware of oral lesions in Darier disease patients (6). The heart, esophagus, stomach, tongue, and any diseased tissues noted during necropsy were removed and examined histologically. Tissues were fixed in 10% neutral buffered formalin, dehydrated through a gradient of alcohols, embedded in paraffin, sectioned, and stained with haematoxylin and eosin.

Western blot analyses—Proteins in tissue homogenates were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and relative SERCA2 levels were determined as described previously (4) using a polyclonal SERCA2 antibody (10) raised against a fusion protein encompassing amino acids 362 to 705 of rat SERCA2, which is common to both SERCA2a and SERCA2b (antibody N1, provided by Jonathan Lytton, Univ. of Calgary). Skin samples were from newborn mice; tongue and forestomach were from 6-, 9-, and 14-month-old mice (one animal of each age).
RESULTS

The original objective of this study was to determine whether the loss of one $Atp2a2$ allele, which impairs cardiac function in young adult mice, would cause heart disease in older animals. We changed our objective when we observed two deaths of unknown causes (autolysis precluded detailed analysis of tissues, although one mouse had a prolapsed penis) among mutant mice of an aging cohort of 16 experimental pairs. The remaining mice were monitored over time and 12 of the 14 $Atp2a2^{+/−}$ mice were examined when disease symptoms were clearly apparent, at ages ranging from 53-81 weeks. The two mutants that did not exhibit overt disease symptoms during the course of the study were euthanized and examined at 89 weeks of age. The wild-type controls, most of which showed no evidence of disease symptoms, were euthanized ~4-5 weeks after the last $Atp2a2^{+/−}$ mice; their ages ranged from 81 to 94 weeks.

As shown in Fig. 1, the viability of $Atp2a2^{+/−}$ mice was markedly decreased compared with that of wild-type mice. Indications of disease among the $Atp2a2^{+/−}$ mice included growths on the face, abdominal masses, prolapsed penis, lethargy, and loss of weight. The benign skin lesions characteristic of Darier disease in humans were not apparent in $Atp2a2^{+/−}$ mice but, as described below, the affected cell type, keratinocytes, was the same. Although mild fibrosis was observed in some of the hearts from diseased $Atp2a2^{+/−}$ mice, there were no major cardiac lesions or hypertrophy when compared with wild-type controls. As summarized in Table 1, the major histopathological correlates of morbidity in $Atp2a2^{+/−}$ mice were hyperkeratinized squamous cell tumors of the stomach, esophagus, tongue, oral mucosa, and skin (13/14 mice, 93%), with an average of more than two tumors per mouse. Two $Atp2a2^{+/−}$ mice exhibited extreme hyperkeratosis of a toenail, and another heterozygote had a large cystic mass containing keratin and bordered by squamous epithelium.
Among the 16 wild-type controls, two males died of unknown causes (at 58 and 86 weeks), a 94-week-old male had a hepatocellular adenoma, a 91-week-old male had an adenocarcinoma of the lung and hyperplasia of the forestomach, and an 82-week-old female had a mammary carcinoma, a leiomyosarcoma of one uterine horn, a lymphoma, and an alveolar adenoma. Squamous cell tumors or evidence of hyperkeratosis or dyskeratosis of the tissues that were affected in heterozygous mutants were not observed in the wild-type mice.

Squamous cell tumors of the forestomach (non-glandular mucosa) and esophagus occurred in 10/14 (71%) \( \text{Atp2a2}^{+/−} \) mice. Six animals had hyperkeratinized squamous cell carcinomas of the forestomach (Fig. 2A-C). Although less common than carcinomas, squamous cell papillomas were also observed in forestomachs of \( \text{Atp2a2}^{+/−} \) mice (Fig. 2D). Papillomas of the esophagus occurred in 7/14 mutant mice (Fig. 2E). Even in the absence of overt tumors of the stomach and esophagus, hyperplastic changes in the epithelial lining of the forestomach and esophagus were observed frequently (Table 1). Squamous cell tumors were observed in the tongue or oral mucosa of 9/10 of the \( \text{Atp2a2}^{+/−} \) mice in which the mouth was examined. Four mice had carcinomas of the tongue (Fig. 2F, G), three had papillomas of the tongue (Fig. 2H), and two had carcinomas of the oral mucosa (cheek) (Fig. 2I). Squamous cell tumors of the skin were seen in 5/14 mice and included two with carcinomas of the face (Fig. 2J, K), two with carcinomas of the penis (Fig. 2L), and one with a papilloma of the penis. We have observed additional squamous cell tumors in retired \( \text{Atp2a2}^{+/−} \) breeders, but not in their wild-type mates; tumors in locations that were not seen in the 14 mutants described in Table 1 included carcinomas of the esophagus, lip, palette, and the skin adjacent to the vagina, penis, and anus.

Western blot analysis of tissue homogenates from unaffected wild-type and \( \text{Atp2a2}^{+/−} \) mice (Fig. 3) showed that SERCA2 protein levels were reduced in skin, tongue, and forestomach of the
mutants (relative to wild-type levels: skin, 66 ± 5%; tongue, 80 ± 2%; forestomach, 82 ± 3%). These data demonstrate that the loss of one \textit{Atp2a2} allele causes a reduction in SERCA2 protein levels in these tissues, as observed previously in heart (4).
DISCUSSION

Our results demonstrate that the loss of one *Atp2a2* allele in mice, which causes reduced expression of the SERCA2 Ca\(^{2+}\) pump, leads to squamous cell tumors of the skin, oral mucosa, esophagus, and forestomach. Most of these tumors are rare in normal mice; among 4900 mice analyzed in the National Toxicology Program, there were only 11 cases of squamous cell tumors of the skin, 4 cases of squamous cell tumors of the oral cavity (any site), and only one squamous cell tumor of the esophagus (11). In the current study, we observed 30 squamous cell tumors among 14 *Atp2a2*\(^{+/−}\) mice and none in the wild-type controls. The high predisposition of *Atp2a2*\(^{+/−}\) mice to the development of squamous cell tumors provides compelling evidence that a perturbation of intracellular Ca\(^{2+}\) homeostasis and/or signaling can lead to cancer.

Squamous cell carcinomas and papillomas are not a common feature of Darier disease, although a number of case histories have been reported (12-14), consistent with the possibility that SERCA2 haploinsufficiency may cause a low incidence of skin cancer in humans. Similarly, although we did not observe skin lesions in the mouse model that resembled the keratotic papules seen in Darier disease, we did observe hyperkeratotic nail abnormalities and a keratinized cyst (Table 1), both of which are reminiscent of findings in Darier patients (6, 15). Further studies will be needed to gain a clearer understanding of the similarities and dissimilarities in disease phenotypes between the two species.

In both mouse and human, lesions resulting from SERCA2 haploinsufficiency arise in keratinized squamous epithelial cells, suggesting that there are similarities in the initial stages of disease in the two species. Although there are species differences in the location of the affected tissues, this is likely to be due to differences in the distribution of keratinized squamous epithelial cells. In mice, this includes the skin, oral mucosa, tongue, esophagus, and forestomach (16), whereas in humans, it includes the skin.
and oral palate, where Darier lesions have been observed (6). In both humans and mice, keratinocytes have been shown to be highly sensitive to perturbations of calcium homeostasis (8, 17-19) and to exhibit changes in differentiation and proliferation in response to treatment with thapsigargin, an inhibitor of SERCA Ca\(^{2+}\) pumps (8). Thus, the \(Atp2a2^{+/}\) mouse should serve as a suitable model for studying the perturbations in Ca\(^{2+}\) homeostasis and signaling in keratinocytes that occur as an immediate consequence of the mutation, and the subsequent changes, including additional genetic mutations, that lead to cancer.

Important issues to be addressed in future studies are the genetic and cell biological mechanisms of tumorogenesis. It is conceivable that the tumors arise from cells that have lost the remaining wild-type allele; however, given the critical function of SERCA2 in maintaining endoplasmic reticulum Ca\(^{2+}\) stores and the severe effect of SERCA2 haploinsufficiency in keratinocytes of Darier disease patients, it seems more likely that the perturbation of Ca\(^{2+}\) homeostasis causes an increased susceptibility to the accumulation of genetic changes at other loci. Some of the immediate consequences of a reduction in SERCA2 activity with respect to intracellular Ca\(^{2+}\) homeostasis and signaling are reasonably well understood (20). Partial inhibition of SERCA2 by exposure to thapsigargin, a tumor promoter in murine two-stage skin carcinogenesis (21), increases the frequency of intracellular Ca\(^{2+}\) spikes in some cells (22). This suggested that an impaired ability to buffer elevations in cytoplasmic Ca\(^{2+}\) by removing it from the cytosol may decrease the interspike interval by stimulating Ca\(^{2+}\)-induced Ca\(^{2+}\) release from the endoplasmic reticulum (22). Depletion of Ca\(^{2+}\) stores by Ca\(^{2+}\) release during signaling events or by treatment with thapsigargin has been shown to cause increased Ca\(^{2+}\) influx across the plasma membrane (23, 24). Interestingly, a Ca\(^{2+}\) influx inhibitor, carboxyamido-triazole, which would appear to have the potential to correct some of the imbalances in Ca\(^{2+}\) homeostasis resulting from mutations in the \(Atp2a2\)
gene (or its human homolog in Darier disease), has been shown to be effective in the treatment of some cancers (9, 25, 26). It is conceivable that it could be used in a topical treatment for Darier disease. Ultimately, the increased cytosolic Ca$^{2+}$ levels and excitability of Ca$^{2+}$ signaling mechanisms occurring as a direct result of SERCA2 haploinsufficiency may cause perturbations in gene expression (24, 27), nuclear Ca$^{2+}$ homeostasis (28), DNA repair (28, 29), and cell cycle regulation (30, 31), with the resulting genetic instability contributing to cancer.
Acknowledgements and footnotes

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1 The abbreviations used are: SERCA, sarco(endo)plasmic reticulum Ca\textsuperscript{2+}-ATPase; SERCA2, SERCA isoform 2; \textit{Atp2a2}\textsuperscript{+/−}, SERCA2 heterozygous mutant.
REFERENCES


LEGENDS

Fig. 1. Survival curves of wild-type and Atp2a2<sup>+/−</sup> mice. Sixteen sex-matched sibling-pairs were aged and their general health was monitored. Mice showing signs of morbidity were euthanized, necropsy was performed, and selected tissues were examined histologically. Each point indicates the percentage of mice that survived and showed no signs of morbidity at that age. Two Atp2a2<sup>+/−</sup> and two wild-type mice died of unknown causes. Histological analyses showed that the Atp2a2<sup>+/−</sup> mice that exhibited signs of morbidity had squamous cell tumors.

Fig. 2. Squamous cell carcinomas and papillomas of keratinized epithelial tissues in Atp2a2<sup>+/−</sup> mice. A, forestomach (FS) carcinoma in a 58-week-old male; the glandular stomach (GS) was intact; the tumor extended into the abdominal cavity with severe inflammation, necrosis and adhesion to adjacent tissues, including spleen (Sp). B, forestomach carcinoma in a 76-week-old male. Inset, gross appearance of stomach, with multiple lesions of forestomach (FS) and no lesions of glandular stomach (GS). C, forestomach carcinoma in a 68-week-old male with extensive keratinization (arrows). D, multifocal papillomas (arrows) in forestomach of a 75-week-old male. E, moderately dysplastic papilloma in esophagus of a 66-week-old male. F, squamous cell carcinoma (SCC) of the tongue of a 75-week-old male; the palette (P) was retracted upwards to show lesion at base of tongue (T). G, carcinoma of the tongue of a 58-week-old male. H, papilloma of the tongue of a 66-week-old male. I, carcinoma of the oral mucosa (cheek) of a 76-week-old male. J, skin carcinoma on the jaw of a 65-week-old female. K, skin carcinoma in large, abscessed lesion from below right ear of a 74-week-old male. L, skin carcinoma on penis of a 66-week-old male. Scale bars: 100 µm (B, E, G, H, K); 200 µm (I); 500 µm (C, D, J); 1 mm (L).
Fig. 3. Western blot analysis of SERCA2 protein levels in tissues of wild-type and heterozygous mutant mice. Varying amounts of protein (0.63-5 µg or 1.25-10 µg, as indicated) from tissue homogenates of wild-type (WT) and heterozygous mutant (HET) mice were analyzed using an anti-SERCA2 antibody (see Experimental Procedures). A, representative blot of samples from skin (n = 3 of each genotype), tongue (n = 3 of each genotype), and forestomach (n = 2 of each genotype). B, amount of SERCA2 protein (mean ± SE) in heterozygous tissues as a per cent of WT controls.
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--- *a*: no lesions observed; *b*: NE-not examined;
Figure 1
Figure 3
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