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Homozygous deletion of *CDKN2A/2B* is a hallmark of iron-induced high-grade rat mesothelioma

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In humans, mesothelioma has been linked to asbestos exposure, especially crocidolite and amosite asbestos, which contain high amounts of iron. Previously, we established a rat model of iron-induced peritoneal mesothelioma with repeated intraperitoneal injections of iron saccharate and an iron chelator, nitrilotriacetate. Here, we analyze these mesotheliomas using array-based comparative genomic hybridization (aCGH) and gene expression profiling by microarray. Mesotheliomas were classified into two distinct types after pathologic evaluation by immunohistochemistry. The major type, epithelioid mesothelioma (EM), originated in the vicinity of tunica vaginalis testis, expanded into the upper peritoneal cavity and exhibited papillary growth and intense podoplanin immunopositivity. The minor type, sarcomatoid mesothelioma (SM), originated from intraperitoneal organs and exhibited prominent invasiveness and lethality. Both mesothelioma types showed male preponderance. SMs revealed massive genomic alterations after aCGH analysis, including homozygous deletion of *CDKN2A/2B* and amplification of *ERBB2* containing region, whereas EMs showed less genomic alterations. Uromodulin was highly expressed in most of the cases. After 4-week treatment, iron deposition in the mesothelia was observed with 8-hydroxy-2′-deoxyguanosine formation. These results not only show two distinct molecular pathways for iron-induced peritoneal mesothelioma, but also support the hypothesis that oxidative stress by iron overload is a major cause of *CDKN2A/2B* homozygous deletion.

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Iron is universally abundant. During evolutionary processes, vertebrates have selected iron as a carrier of oxygen (hemoglobin) inside the body. However, iron represents a double-edged sword as excess levels increase the risk for cancer, presumably through the generation of reactive oxygen species (ROS).[1] Thus far, pathological conditions such as those resulting from asbestos fiber exposure as well as hemochromatosis, chronic viral hepatitis B and C, and endometriosis have been recognized as iron overload-associated risks for human cancers.[2]

Respiratory exposure to asbestos fibers has been associated with diffuse malignant mesothelioma in humans. Despite advancements in the molecular analysis of human mesothelioma and in the development of animal models, the carcinogenic mechanisms of the disease remain unclear. Mesothelioma with poor prognosis continues to be a serious social problem in many countries.[3] Therefore, it is important to elucidate the carcinogenic mechanisms of mesothelioma in order to establish techniques for early diagnosis and to develop preventive strategies for people exposed to asbestos. Currently, three hypotheses exist regarding the pathogenesis of asbestos-induced mesothelioma.[4] The 'oxidative stress theory'[5] is based on the fact that fibrous mineral foreign substances in asbestos are phagocytosed by macrophages, which are unable to digest them, resulting in the massive generation of ROS. Alternatively, crocidolite and amosite may present catalyzing reactive environments with exposed surface iron. Consistent with this hypothesis, epidemiological studies indicate that asbestos fibers containing iron are more carcinogenic.[6] The 'chromosome tangling theory' postulates that asbestos fibers damage chromosomes when cells divide.[7] Finally, the 'molecular adsorption theory' states that a variety

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of proteins and chemicals including components of cigarette smoke bind to the broad surface area of asbestos.[5] This might result in the accumulation of hazardous molecules.

The first hypothesis stresses the importance of local iron overload in the carcinogenic process of mesothelioma. In 1989, we succeeded in producing a rat model of peritoneal mesothelioma using ferric saccharate followed by administration of an iron chelator, nitrilotriacetate. This model demonstrated, for the first time, that local iron deposition is an important factor in the generation of diffuse malignant mesothelioma.[8] In this study, we have applied two microarray techniques to these iron-induced peritoneal mesotheliomas to elucidate the molecular mechanisms of iron-induced mesothelial carcinogenesis.

MATERIALS AND METHODS

Animal Experiments and Chemicals

The carcinogenesis study was performed as previously described[8] with slight modification, using specific pathogen-free F1 hybrid rats crossed between Fischer344 (F344; female) and Brown-Norway (BN/CIJ; male) strains (Charles River, Yokohama, Japan). In some acute and subacute experiments, specific pathogen-free male Wistar rats (Shizuoka Laboratory Animal Center, Shizuoka, Japan) were also used. Animals were fed a basal diet (Funabashi F-1; Funabashi, Chiba, Japan) and tap water *ad libitum*. Ferric saccharate (Fesin; Yoshitomi Pharmaceutical Company, Osaka, Japan) was prepared in a 5% glucose solution. The nitrilotriacetic acid (NTA) solution was prepared by dissolving NTA, disodium salt (Nakalai Tesque, Kyoto, Japan), in physiological saline, and the pH was adjusted using sodium bicarbonate to pH 7.4. For the carcinogenesis study, a total of 92 F1 hybrid rats were divided into two groups of untreated control ($N=38$) and iron-treated ($N=54$). The injections were started at 4 weeks of age. Ferric saccharate (5 mg iron/kg body weight) was injected intraperitoneally 5 days a week for 12 weeks. NTA (80 mg/kg body weight) was administered separately intraperitoneally 5 days a week for 20 weeks. This form of iron primarily deposits in the peritoneum.[8] The animals were kept under close observation and were killed when they showed persistent weight loss and/or distress. The experiments were terminated at 26.7 months of age. All the animals were autopsied. Samples were either immediately frozen and stored at $-80^{\circ}C$ until use, or subjected to routine histological analysis with buffered 10% formalin fixation and paraffin embedding. The animal experiment committees of the Graduate School of Medicine, Kyoto University and Nagoya University Graduate School of Medicine approved these animal experiments. All chemicals used were of analytical quality.

Rat Peritoneal Mesothelial Cells

Rat peritoneal mesothelial cells (RPMC) were cultured from the omentum of Wistar rats as previously described,[9] and grown in RPMI 1640 medium containing 10% fetal calf serum. A retroviral vector pCMSCVpuro-16E6E7 was constructed by recombining the segment of a donor vector containing full-length HPV16E6 and E7 (A kind gift from Dr Tohru Kiyono, National Cancer Center, Tokyo, Japan) into the destination vector by the Gateway System (Invitrogen Life Technologies, Carlsbad, CA, USA) as described previously.[10] RPMCs were infected at day 14 by the recombinant retrovirus expressing the 10A1 envelope[11] with 4 μg/ml polybrene, and were drug selected using 1 μg/ml puromycin.

Array-Based CGH Analysis

We performed array-based comparative genomic hybridization (aCGH) with a Rat Genome CGH Microarray 244A (G4435A; Agilent Technologies, Santa Clara, CA, USA), as described in the Agilent Oligonucleotide Array-based CGH for Genomic DNA Analysis Protocol ver. 5.0, and analyzed results with DNA Analytics Software (ver. 4.0). For each array, normal kidney was used as a reference and labeled with Cy-3. Samples of interest were labeled with Cy-5. Six cases of epithelioid mesotheliomas (EMs) and five cases of sarcomatoid mesotheliomas (SMs) were analyzed.

Fluorescent *In Situ* Hybridization

Appropriate bacterial artificial chromosome probes were selected from http://genome.ucsc.edu/ and purchased from http://bacpac.chori.org/. CH230-163D24 was used for *CDKN2A/2B*, and CH230-209G15 for *Erbb2*. Fluorescent probes were labeled by incorporating Green-dUTP (Vysis; Abbott Laboratories; Abott Park, IL, USA) into newly synthesized DNA by the Nick Translation Kit (Vysis). Fluorescent *in situ* hybridization (FISH) was performed using the probes, Paraffin Pretreatment Kit and LSI/WCP Hybridization Buffer (Vysis) according to the manufacturer's protocol. Briefly, paraffin sections were treated with protease, and after denaturation, the probes were hybridized to nuclear DNA, counterstained with DAPI, and visualized using a fluorescence microscope.

Expression Microarray Analysis

A total of 12 microarrays (Whole Rat Genome Microarray, G4131F; Agilent Technologies) were used for the screening purpose: four chips were used for tooth brush-scraped pleural and peritoneal mesothelial cells and soft tissue surrounding tunica vaginalis testis, six for EMs and two for SMs. Total RNA was isolated with an RNeasy Mini kit (QIAGEN GmbH, Hilden, Germany). Data analysis was performed using GeneSpring GX 10.02.2 software (Agilent Technologies).

Quantitative RT-PCR Analysis

Total RNA was extracted with RNeasy Mini (QIAGEN) and cDNA was synthesized using SuperScript III First-strand Synthesis System for RT-PCR (Invitrogen Life Technologies) with random primers. All of the primers used are summarized in the Supplementary Table 1.

Antibody

An anti-uromodulin rabbit polyclonal antibody was produced by a commercial supplier (Hokudo, Hokkaido, Japan). Briefly, a 15-mer polypeptide (NH_3-CKQDFNVTDVSLLEH-COOH) corresponding to the 320-334 cytoplasmic portion of rat UMOD protein (AAH81814) was synthesized and conjugated with keyhole limpet hemocyanin, which was used as an immunogen for JW rabbits. Five weeks after the second immunization, whole serum was harvested and purified using a SulfoLink Kit (Pierce, Rockford, IL, USA). Anti-S-100 polyclonal antibody (LSL-LB-9197) was from Cosmo Bio (Tokyo, Japan). Anti-desmin monoclonal antibody (clone D33) was from DAKO (Carpinteria, CA, USA). Anti-podoplanin polyclonal antibody (KS-17) was from Sigma (Saint Louis, MO, USA). Anti-multi-cytokeratin monoclonal antibody (RTU-AE1/AE3) was from Novocastra (Newcastle, UK). Anti-8-hydroxy-2′-deoxygunosine monoclonal antibody (clone N45.1)[12] was from Nikken Seil (Shizuoka, Japan). Anti-single stranded DNA antibody (no. 18731) was from IBL (Takasaki, Gunma, Japan).

Western Blot Analysis

This was performed using a standard procedure as previously described.[13,14]

Histology, Tissue Array, and Immunohistochemical Analysis

The specimens embedded in paraffin were cut at 3-μm thickness, stained with hematoxylin and eosin, or used for immunohistochemistry. Representative areas were chosen and cores of 3 mm diameter were punched out from the blocks with a precision instrument (Tissue Microprocessor; Azumaya, Tokyo, Japan). Those cores of 24 (6×4 array) in a group were embedded in a paraffin block. Immunohistochemistry was performed as previously described.[12] Antigen retrieval for single-stranded DNA was by incubation with proteinase K solution (Trevigen, Gaithersburg, MD, USA) at 37°C for 30 min. Negative controls are shown in the Supplementary Figure 1.

Detection of DNA-Strand Breaks

TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; TACS 2 TdT-Blue Label *In situ* Apoptosis Detection kit; Trevigen) method and immunohistochemical analysis for single-stranded DNA were used.

Statistical Analysis

Statistical analyses were performed with an unpaired *t*-test, which was modified for unequal variances when necessary. Kaplan–Meier analysis was also used. $P < 0.05$ was considered as statistically significant.

RESULTS

Two Distinct Pathologies were Observed in Ferric Saccharate-Induced Rat Peritoneal Mesothelioma

F1 hybrids between Fischer344 and Brown-Norway rat strains were used to allow allele-specific analyses when necessary. There was no significant difference in the survival between the control and iron-injected groups up to 20 months of age. Thereafter, animals in the iron-treatment group started to die from mesothelioma (Figure 1). Control animals generated no mesothelioma during the 26.7-month postnatal observation period. All observed tumors are summarized in Tables 1 and 2.

Two distinct pathologies were observed in the obtained mesotheliomas. The majority of tumors were observed in the vicinity of tunica vaginalis testis, and expanded to the upper peritoneal cavity in half of the cases (Figure 2). Most of the animals did not die from the disease during the observation period. When fine tumors were scattered throughout the whole peritoneum, we recognized these as malignant. Such tumors revealed papillary growth patterns and were always intensely positive for podoplanin and cytokeratin (Figure 2). These tumors were counterparts of human EM.

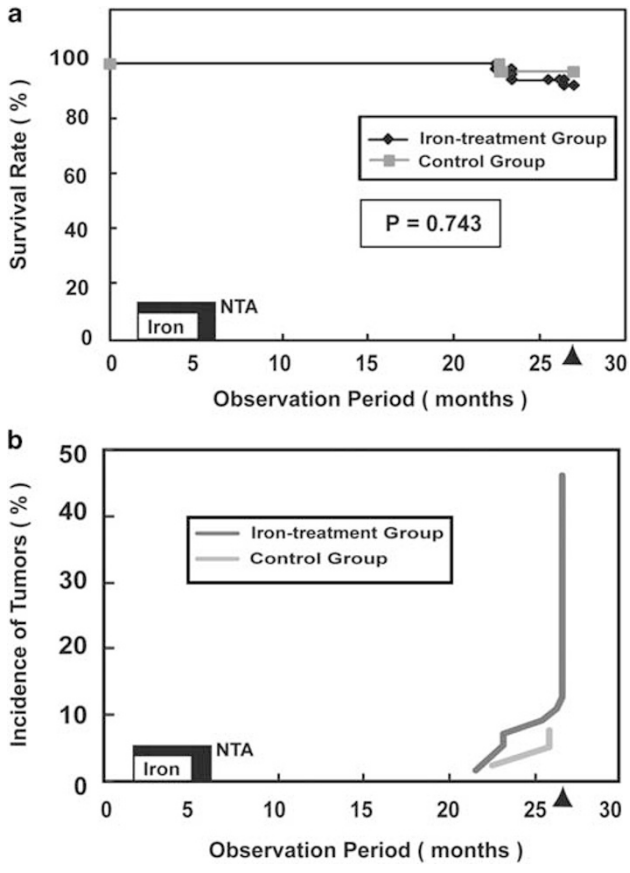


Figure 1 Survival rates (**a**) and incidence of tumors (**b**) in the iron-treatment group (iron saccharate followed by nitrilotriacetic acid) and the untreated control group. The arrow head shows the point of experiment termination (26.7 months after birth). NTA, nitrilotriacetic acid. Refer to Materials and methods section for details.

Table 1 Incidence of peritoneal mesothelioma by intraperitoneal injections of ferric saccharate and nitrilotriacetic acid

	Number of rats with mesothelioma	Number of rats with other tumors
Iron-treatment group		
Male ($N=24$)	16 (66.7%)	1 (4.2%)
Female ($N=30$)	1 (3.3%)	7 (23.3%)
Control group		
Male ($N=17$)	0 (0%)	2 (11.8%)
Female ($N=21$)	0 (0%)	1 (4.8%)

Numbers in parentheses show the animal numbers used in each group.

A second tumor type was found associated with intra-abdominal organs such as the stomach, spleen, and abdominal wall (Figure 2). These tumors showed strong invasive activity and sometimes presented as a mass involving several organs, omentum, and mesentery. Because of their invasive nature, these tumors were lethal primarily due to intestinal obstruction. Ascites was not observed. Histology showed high-grade spindle cell tumors, proliferating on the serosal surface of the organs. By immunohistochemical analysis, these tumors were cytokeratin-negative, desmin-negative but podoplanin-positive (weak to moderate). Immunohistochemistry for S-100 was sometimes weakly positive (Figure 2). Thus, these tumors represented counterparts of human SM. No transition from EM to SM was observed. Both tumor types, EM and SM, presented male preponderance (Table 1).

Array-Based CGH Analyses Classified Ferric Saccharate-Induced Rat Peritoneal Mesothelioma into Two Distinct Types

Six cases of EM and five cases of SM were analyzed by aCGH. Array-based CGH analysis clearly distinguished the two histological types. Although EMs showed numerous minor genomic amplifications and deletions, most of SMs showed a variety of chromosomal amplifications and deletions (Figure 3a). In particular, four out of the five cases of SM showed homozygous deletion of CDKN2A/2B, and in two cases amplification of ERBB2-containing region. The results obtained by aCGH were confirmed by FISH analysis (Figure 3b and c).

Expression Array Analysis Revealed Overexpression of Uromodulin in the Majority of Cases

Six cases of EM and two cases of SM were analyzed with expression microarray analyses (Table 2). The remaining cases of SM were not assayed by this technique because of poor-quality RNA, as the animals were found dead. Expression data for the 20 most strongly up- or downregulated genes are summarized in Tables 3 and 4 (Gene Expression Omnibus accession number GSE16138). We focused on uromodulin in this study as it is a major urinary protein,[15] and mesothelial cells and renal tubular cells are both of mesodermal origin. We confirmed the overexpression of this gene by RT-PCR analysis, western blot analysis, and immunohistochemistry (Figure 4). Brain, kidney, and testis in adult rats showed high expression of uromodulin.

Expression of Mesoderm-Specific Transcription Factors

Several mesoderm-specific early embryogenesis transcription factors were studied along with ectoderm- or endoderm-specific transcription factors. Out of the transcription factors examined, mesotheliomas showed activated DLX5,[16] ONECUT1 (HNF6),[17] and Pax6,[18] whereas the activation of HAND1,[19] ISL1,[20] and MEIS1[21] were not observed.

Oxidative Stress in Peritoneal Mesothelial Cells after Injection of Ferric Saccharate

Prominent iron deposition was found in the mesothelia and surrounding tissue including macrophages. Nuclear immunopositivity for 8-hydroxy-2′-deoxyguanosine (8-OHdG),[12] an oxidatively modified DNA product, was significantly increased in the mesothelia 4 weeks after repeated iron saccharate administration (Figure 5). At the same time, significant increase in DNA-strand breaks of the nuclear genomic DNA were observed in the mesothelia after iron treatment, based on TUNEL method (data not shown) and immunohistochemical analysis for single-stranded DNA (Figure 5).

DISCUSSION

In this study, we followed the established protocol to generate iron-induced peritoneal mesothelioma,[8,22] and analyzed the generated tumors using two different microarray techniques. An iron chelator, nitrilotriacetate, has been used in this fiber-unassociated mesothelioma model to mobilize the catalytic form of iron from deposits.[23,24] Our study revealed a number of important implications regarding mesothelioma, which is a serious social problem in many countries following asbestos exposure. From an epidemiological standpoint, asbestos fibers, in particular, crocidolite and amosite, are recognized to be more carcinogenic when containing high amounts of iron.[6] During the carcinogenenic experiments presented here, a relatively high amount of iron (a total of approximately 300 mg iron/kg body weight) was used. In contrast, only 15 mg iron/kg in the case of crocidolite or amosite is enough to produce high-grade mesothelioma in the majority of rats (Li J and Toyokuni S, unpublished data). Therefore, ferric saccharate is a much weaker carcinogen than crocidolite or amosite, considering the fact that most of the animals did not die from the disease up to 26.7 months. This suggests that the shape, size, and probably surface characteristics of the molecules are important contributing factors in mesothelial

Table 2 Pathological findings of all the induced tumors

Case	Original number	Cell type	Structure	Size of main tumor (mm)	Primary organ	Dissemination range (mm)
M1-male	1-1-male	EM	Papillary	<2 mm, multiple	Testis and peritoneum	120
M2-male	15-1-male	EM	Papillary	<2 mm, multiple	Testis	60
M3-male	15-2-male	EM	Papillary	<3 mm, multiple	Testis	30
M4-male	15-4-male	EM	Papillary	<3 mm, multiple	Testis	30
M5-male	15-5-male	EM	Papillary	<3 mm, multiple	Testis and peritoneum	120
M6-male	13-2-male	EM	Papillary	<2 mm, multiple	Testis and peritoneum	120
M7-male	10-4-male	SM	Solid	25	Stomach and spleen	NA
M8-female	12-4-female	SM	Solid	30	Abdominal wall	NA
M9-male	11-2-male	SM	Solid	50	Stomach	NA
M10-male	13-1-male	SM	Solid	50	Abdominal wall	NA
M11-male	13-2-2-male	SM	Solid	100	Stomach and omentum	150
M12-male	8-2-male	EM	Papillary	<1 mm, multiple	Testis	20
M13-male	8-3-male	EM	Papillary	<1 mm, multiple	Testis	30
M14-male	10-2-male	EM	Papillary	<1 mm, multiple	Testis	<5 mm
M15-male	10-3-male	EM	Papillary	<1 mm, multiple	Testis	<5 mm
M16-male	11-1-2-male	EM	Papillary	<1 mm, multiple	Testis	<5 mm
M17-male	13-3-male	EM	Papillary	<1 mm, multiple	Testis	10
T1-female	M-2-1	Fibroadenoma	Solid	30	Mammary gland	NA
T2-female	M-2-4	Fibroadenoma	Solid	50	Mammary gland	NA
T3-female	M-4-1	Fibroadenoma	Solid	40	Mammary gland	NA
T4-female	M-4-3	Fibroadenoma	Solid	80	Mammary gland	NA
T5-female	M-6-2	Fibroadenoma	Solid	100	Mammary gland	NA
T6-female	M-7-1	Fibroadenoma	Solid	40	Mammary gland	NA
T7-female	M-14-2	Mature cystic teratoma	Solid	50	Ovary	NA
T8-male	O-10-1	Leydig cell tumor	Papillary	6	Testis	NA
C1-male	O-17-4	Hepatocellular carcinoma	Solid	50	Liver	NA
C2-male	O-21-3	Fibrolipoma	Solid	80	Soft tissue	NA
C3-female	M-22-4	Fibroadenoma	Solid	10	Mammary gland	NA

EM, epithelioid mesothelioma; SM, sarcomatoid mesothelioma; NA, not applied.

M1-11 and M1-8 were used for array-based comparative hybridization analyses and expression microarray analyses, respectively.

carcinogenesis. Here, paradoxically the weaker carcinogenesis model did present several novel viewpoints.

First, these experiments revealed a clear preference for tumorigenesis in males. This observation is consistent with the accumulated human epidemiological data.[25] However, in the case of humans, differences in frequency and doses of asbestos exposure between males and females must be considered. Our results indicate that sex hormone and/or anatomical differences such as the presence of tunica vaginalis are also contributing factors in the generation of mesothelioma.

We classified mesotheliomas into two types: low-grade and high-grade. Low-grade tumors were of the epithelioid type,[26] originating from the vicinity of tunica vaginalis testis. Some of these tumors disseminated to the whole abdominal cavity. High-grade tumors were of the sarcomatoid type, originating in the upper abdominal cavity. Fischer-344 strain rats are known to generate testicular mesothelioma albeit at a low incidence (0–1.3%).[27,28] To control this situation, we have used F1 hybrid rats crossed between Fischer-344 and Brown-Norway strains, and the hybrid animals in these experiments presented no mesothelioma in the untreated control group. Therefore, we believe that our data was not affected significantly by the background of genetic susceptibility to mesothelioma.

We confirmed the histological diagnoses by immunohistochemistry in a similar manner as applied to human cases

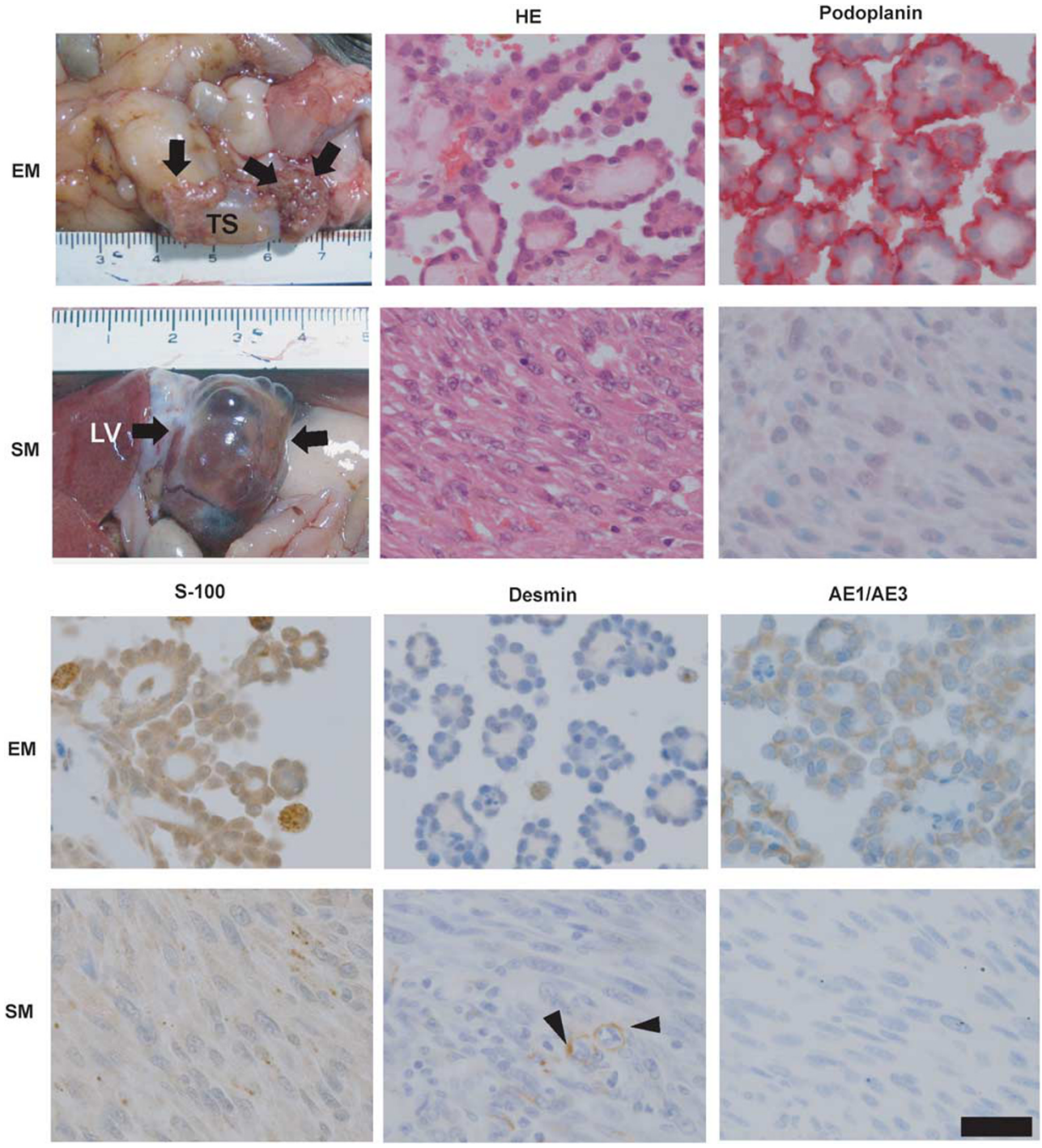


Figure 2 Two distinct types of mesothelioma induced by iron saccharate and nitrilotriacetic acid. EM, epithelioid-type. SM, sarcomatoid-type. Podoplanin was immunostained with red color, whereas S-100, desmin, and pancytokeratin are shown in brown. The arrows indicate mesothelioma. TS, testis. LV, liver. Arrowheads, smooth muscle in vessels (bar = 100 μm).

of mesothelial tumors.[26] Epithelioid types were strongly positive for podoplanin, and sarcomatoid types were negative for desmin (myogenic marker). Unfortunately, many antibodies are not available for rats. Sarcomatoid tumor types showed weak-to-moderate positivity for podoplanin and no positivity for pan-keratin in our study. Some of the cases showed weak positivity for S-100. Such cases are also described in human mesotheliomas.[26] Of note was the fact that these two types of tumors were clearly different in their aCGH profiles. Importantly, most of the sarcomatoid type tumors examined showed homozygous deletion of CDKN2A/2B ($p16^{INK4A}$/$p15^{INK4B}$) with two cases of *ERBB2* amplification.

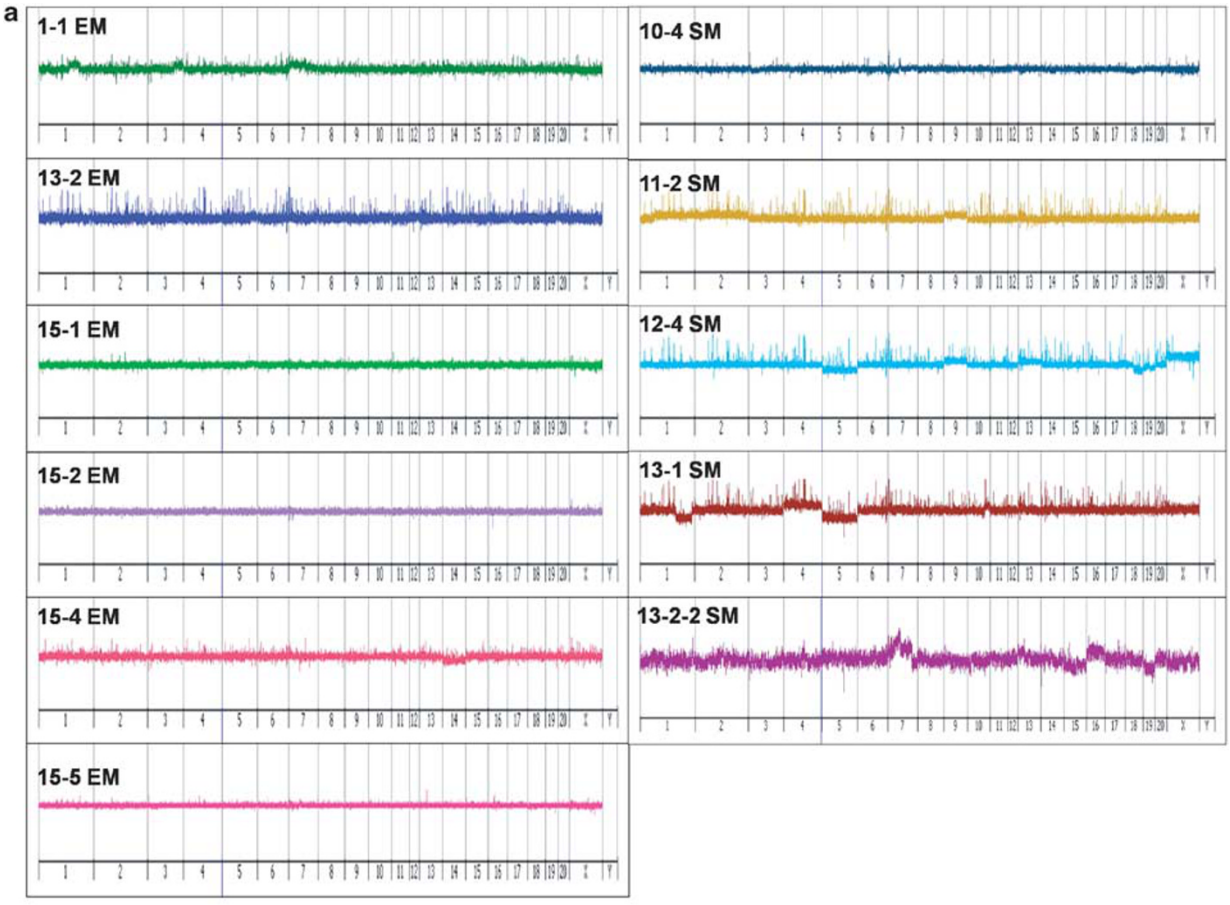
ERBB2 is a receptor tyrosine kinase for epidermal growth factor.[29,30] Activation of *ERBB2* is reported in human mesothelioma cell lines.[31] Our present study demonstrated that oxidative stress is a cause of *ERBB2* amplification. Inactivation of *CDKN2A/2B* is the second most common genetic event in human cancers next to *p53* tumor suppressor gene alterations.[32] There are three different mechanisms of inactivation: (i) homozygous deletion, (ii) inactivating mutation with loss of heterozygosity, and (iii) methylation of the CpG island promoter region. It is probably not a coincidence that *CDKN2A/2B* is a major tumor suppressor gene target in ferric nitrilotriacetate (Fe-NTA)-induced rat renal carcinogenesis model,[33] in which a major mode of inactivation is also homozygous deletion.[34,35] In the renal carcinogenesis model, an iron-catalyzed Fenton reaction is repeatedly induced in the target renal proximal tubular cells early in carcinogenesis[36–38] These data strongly suggest that iron-mediated oxidative DNA damage is a major cause of the homozygous deletion of *CDKN2A/2B*. Interestingly, potassium bromate shares with iron compounds the ability to cause not only mesothlioma[28] but also renal cell carcinoma [39] presumably by oxidative stress.[40]

For the homozygous deletion to occur, DNA double-strand breaks (DSBs) should be present, either as DNA damage or endogenous mechanisms. The presence of DSBs in the genomic DNA during replication is expected to result in homozygous deletion. So far, it is established that γ-radiation, ultraviolet radiation, and transition metals are causative agents of DNA DSBs.[41,42] Furthermore, various repair processes of DNA base modifications.[43] or hypermutation with increased activity of activation-induced deaminase can be endogenous causes of DSBs.[44–46] In many cases of human leukemias, which can be induced by γ-radiation,[47] translocations and deletions are frequently observed.[48,49] However, as far as we know in animal carcinogenesis models, massive chromosomal alterations as seen in the high-grade mesothelioma and Fe-NTA-induced renal cell carcinoma[14] have not been reported, except for genetically engineered mice leading to malignant lymphoma.[50] These results emphasize the importance of iron-mediated oxidative stress in carcinogenesis. Indeed, at the evaluation of the peritoneum 4 weeks after the start of the experiment, iron deposition was clear in the mesothelial cells with evidence of oxidative stress and DNA-strand breaks. Previously, we demonstrated *in vitro* that 8-OHdG formation and single/double-strand breaks in DNA are proportional.[51] 8-OHdG is produced either by $^{\cdot}OH$, $^1O_2$, or photodynamic action. Iron is closely associated with $^{\cdot}OH$ generation as a catalyst in Fenton reaction,[52] and provides a mutation-prone environment that stochastically culminates in tumorigenesis. Thus, the iron-induced animal carcinogenesis presents an ideal model how *CDKN2A/2B* is selected as a target of homozygous deletion in a situation where DNA DSBs and *CDKN2A/2B* are associated in terms of a senescence-like condition.[53] We are currently working on how specific amplification and deletion in the genome are generated by the use of a methodology called oxygenomics.[54]

Next, we would like to discuss how the two different types of mesothelioma are generated. Interestingly, EMs were produced at the lowest point of the body in male animals. It is possible that iron deposition is more prominent around tunica vaginalis testis in males. In contrast, special unidentified mesothelial cells prone to transformation may be present in this anatomical location. In human pleural mesotheliomas, homozygous deletion of *CDKN2A/2B* is present in a majority of cases (69% in EMs and 100% in SMs).[55] This implies that the EMs in our study with no prominent chromosomal instability are at early stages and that deletion of *CDKN2A/2B* is not always necessary for the induction of EMs. On the contrary, all the SMs occurred at the upper abdominal area, concomitant with homozygous deletion of *CDKN2A/2B* in 80% of the cases. Although iron overload appears important to generate the homozygous deletion of *CDKN2A/2B*, we suspect that increased oxygen tension by repeated intraperitoneal injections at the upper abdominal area might have assisted this genomic alteration as oxygen tension of the peritoneal cavity is maintained much lower than the atmosphere.[56] Another contributing factor could be abundant adipocytes in the omentum and mesentery. It is possible that secretion of a variety of cytokines by adipocytes and inflammatory macrophages may have modulated carcinogenesis after iron overload.[57] It also remains elusive whether mesothelioma truly originates from surface mesothelial cells, as mesothelial cells and lymphatic cells are seamlessly connected in the parietal pleura.[58]

In expression microarrays, it is important to choose appropriate control samples. In the case of mesothelioma, this is not trivial. We have tried two different samples, namely, brush-scraped surface cells from the pleural and abdominal cavity and soft tissue surrounding the tunica vaginalis testis, and the average was used. Following expression profiling, we focused primarily on uromodulin and transcription factors associated with early mesodermal differentiation. Uromodulin showed the most significant change, especially in EMs. Uromodulin has been recognized as the most

Figure 3 Array-based comparative genome hybridization analysis of mesotheliomas. (**a**) Whole genome data. EM, epithelioid-type. SM, sarcomatoid-type. Numbers denote the rat chromosome number. Sarcomatoid-type tumors revealed more extensive chromosomal alterations than epithelioid-type tumors. (**b**) Data from the long-arm of chromosome 5 in sarcomatoid mesothelioma. Common homozygous deletion of *p16* (*CDKN2A*) and *p15* (*CDKN2B*) is observed. Inset shows the magnification of *CDKN2A/2B* area (longitudinal line shows the exact gene location). Representative data from FISH analysis are shown (orange signals under DAPI nuclear counterstaining; left, splenic lymphocytes; right, mesothelioma). (**c**) Data from the long-arm of chromosome 10 in sarcomatoid mesothelioma. Common amplification of *ERBB2* is observed. Inset shows the magnification of *ERBB2* area (longitudinal line shows the exact gene location). Representative data from FISH analysis are shown (green signals under DAPI nuclear counterstaining; two mesotheliomas with or without *ERBB2* amplification).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1-1 EM
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13-2 EM
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15-1 EM
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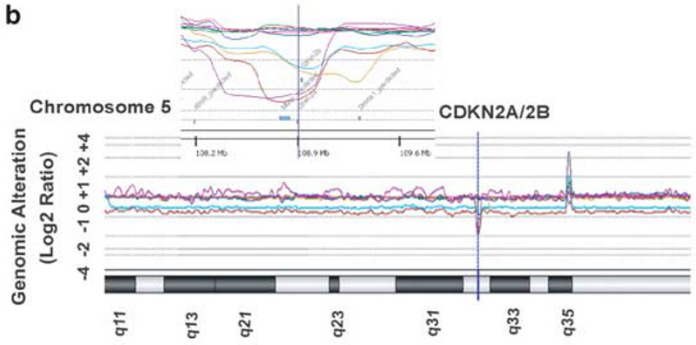
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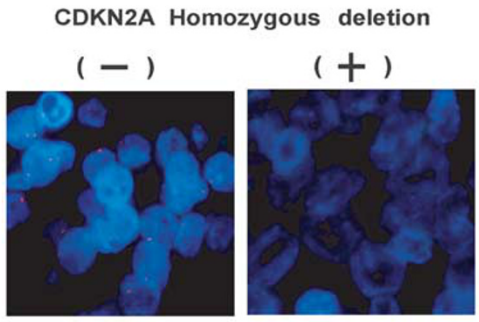
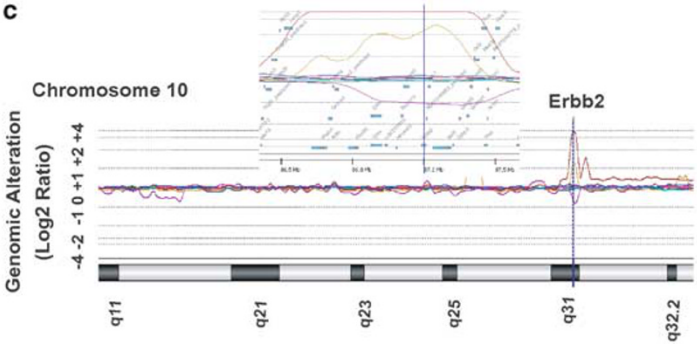
q33
q35


CDKN2A Homozygous deletion
(—)
(+)


c
Chromosome 10
Erbb2
Genomic Alteration
(Log2 Ratio)
-4 -2 -1 0 +1 +2 +4
q11
q21
q23
q25
q31
q32.2

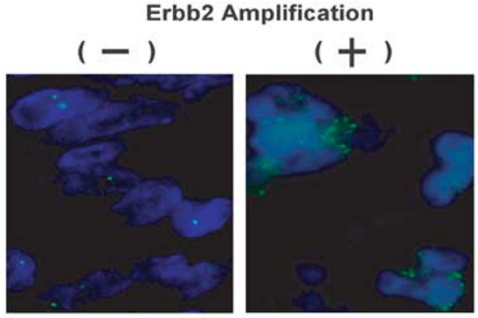

Erbb2 Amplification
(—)
(+)

Table 3 Top 20 Upregulated genes in iron-induced mesothelioma

GenBank no.	Gene description	Fold change (log2)
Genes upregulated	Epithelioid mesothelioma	
NM_001017496	Similar to small inducible cytokine B13 precursor (CXCL13)	9.218209417
NM_001008831	RT1 class II, locus Ba (RT1-Ba)	8.71250575
NM_017082	Uromodulin (Umod)	7.9219705
NM_022175	Placentae and embryos oncofetal gene (Pem)	7.658948067
U15550	Tenascin-C	7.634158617
NM_012881	Secreted phosphoprotein 1 (Spp1)	7.575371133
NM_024142	Matrix extracellular phosphoglycoprotein with ASARM motif (bone) (Mepe)	7.260534987
NM_001008560	Protease, serine, 35 (Prss35)	7.201287705
NM_013028	Short stature homeobox 2 (Shox2),	7.152587133
NM_017216	Solute carrier family 3, member 1 (Slc3a1)	7.004159133
NM_031757	Matrix metallopeptidase 24 (Mmp24)	6.9717793
NM_030837	Kidney-specific organic anion transporter (Slc21a4)	6.9573312
NM_001024893	Similar to melanoma antigen family A, 5 (MGC114427)	6.673916917
NM_031044	Histamine N-methyltransferase (Hnmt)	6.611724693
NM_017210	Deiodinase, iodothyronine, type III (Dio3)	6.5584509
ENSRNOT00000020919	Glia-derived nexin precursor (GDN) (Protease nexin I) (PN-1)	6.543974733
NM_019134	Solute carrier family 12, member 1 (Slc12a1)	6.519795817
NM_012995	Oncomodulin (Ocm)	6.427419253
NM_175760	Cytochrome P450, family 4, subfamily a, polypeptide 14 (Cyp4a14)	6.383245517
NM_022590	Solute carrier family 5 (sodium/glucose cotransporter), member 2 (Slc5a2)	6.297669483
Genes upregulated	Sarcomatoid mesothelioma	
NM_001008560	Protease, serine, 35 (Prss35)	8.482390353
ENSRNOT00000003452	Tenascin N (predicted) (Tnn_predicted)	8.014980133
NM_001008831	RT1 class II, locus Ba (RT1-Ba)	7.632204375
NM_013028	Short stature homeobox 2 (Shox2)	7.028548317
ENSRNOT00000056534	Fragile X mental retardation 1 neighbor (Fmr1nb_predicted)	7.009716683
NM_133523	Matrix metallopeptidase 3 (Mmp3)	6.860669808
ENSRNOT00000031175	Cellular retinoic acid-binding protein 1(CRABP-I)	6.7431448
U15550	Tenascin-C	6.739385108
NM_012881	Secreted phosphoprotein 1 (Spp1)	6.479985417
NM_019282	Gremlin 1 homolog, cysteine knot superfamily (Xenopus laevis) (Grem1)	6.262645658
NM_172333	Collagen triple helix repeat containing 1 (Cthrc1)	6.101469273
ENSRNOT00000020919	Glia-derived nexin precursor (GDN) (Protease nexin I) (PN-1)	5.882276717
NM_001006993	Sarcoglycan, gamma (dystrophin-associated glycoprotein) (Sgcg)	5.860486633
ENSRNOT00000011507	Collagenase 3 precursor (EC 3.4.24.-) (Matrix metalloproteinase-13) (MMP-13)	5.846046783
NM_053881	Protein tyrosine phosphatase, receptor type, N (Ptprn)	5.760772425
NM_021666	Triadin (Trdn)	5.510140083
NM_012980	Matrix metallopeptidase 11 (Mmp11)	5.505010658
NM_013046	Thyrotropin releasing hormone (Trh)	5.49094599
NM_001025155	Leucine-rich repeat containing 17 (Lrrc17)	5.43975145
NM_013153	Hyaluronan synthase 2 (Has2)	5.407057475

Table 4 Top 20 Downregulated genes in iron-induced mesothelioma

GenBank no.	Gene description	Fold change (log2)
Genes downregulated	Epithelioid mesothelioma	
ENSRNOT00000006885	PREDICTED: similar to cystatin E2	−8.73490455
NM_138842	Surfactant-associated protein B (Sftpb)	−8.402350217
NM_138854	Solute carrier family 38, member 5 (Slc38a5)	−7.91033265
NM_053983	CD52 antigen (Cd52)	−7.259246867
XM_574039	PREDICTED: glutathione peroxidase 5 (Gpx5)	−7.182397383
NM_153301	Arachidonate 15-lipoxygenase, second type (Alox15b)	−7.109294617
NM_139085	Cystatin 11 (Cst11)	−7.021647783
NM_144744	Adiponectin, C1Q and collagen domain containing (Adipoq)	−6.90978975
NM_001012056	Carboxylesterase 615 (LOC307660)	−6.658661367
NM_001001519	Lipocalin 6 (Lcn6)	−6.620507583
NM_001009524	Beta-galactosidase-like protein (Bin2a)	−6.5667172
NM_139339	Tramdorin 1 (Slc36a2)	−6.48272195
ENSRNOT00000044284	PREDICTED: lipocalin 9 (predicted) (Lcn9_predicted)	−6.467987617
NM_019258	Cystatin 8 (cystatin-related epididymal spermatogenic) (Cst8)	−6.3628091
NM_017342	Surfactant-associated protein C (Sftpc)	−6.166993217
NM_001008561	Ribonuclease, RNase A family, 9 (non-active) (Rnase9)	−5.987007817
NM_134326	Albumin (Alb)	−5.957892917
NM_053730	Stromal antigen 3 (Stag3)	−5.949726633
NM_001001934	Lymphocyte antigen 6 complex, locus G5B (Ly6g5b)	−5.9309187
NM_153734	Cystatin TE-1 (LOC266776)	−5.847135583
Genes downregulated	Sarcomatoid mesothelioma	
NM_138842	Surfactant-associated protein B (Sftpb)	−9.550074108
NM_053983	CD52 antigen (Cd52)	−8.859255933
ENSRNOT00000006885	PREDICTED: similar to cystatin E2	−8.451462275
XM_574039	PREDICTED: glutathione peroxidase 5 (Gpx5)	−8.209176692
NM_138854	Solute carrier family 38, member 5 (Slc38a5)	−7.371380075
NM_139085	Cystatin 11 (Cst11)	−7.356058742
NM_001001519	Lipocalin 6 (Lcn6)	−6.846685092
NM_153301	Arachidonate 15-lipoxygenase, second type (Alox15b)	−6.697708308
NM_001009524	Beta-galactosidase-like protein (Bin2a)	−6.5989881
NM_001009540	Tumor-associated calcium signal transducer 2 (Tacstd2)	−6.56866565
NM_001008561	Ribonuclease, RNase A family, 9 (non-active) (Rnase9)	−6.462160908
NM_001012056	Carboxylesterase 615 (LOC307660)	−6.120110183
NM_001004236	Tetraspanin 1 (Tspan1)	−6.071135933
NM_019258	Cystatin 8 (cystatin-related epididymal spermatogenic) (Cst8)	−6.01918205
NM_147213	Alpha-2u globulin PGCL5 (LOC259245)	−6.012992958
NM_139339	Tramdorin 1 (Slc36a2)	−5.990390975
ENSRNOT00000044284	PREDICTED: lipocalin 9 (predicted) (Lcn9_predicted)	−5.982269108
NM_001006990	Cell adhesion molecule JCAM (LOC304000)	−5.772606325
NM_001001934	Lymphocyte antigen 6 complex, locus G5B (Ly6g5b)	−5.756341
NM_053730	Stromal antigen 3 (Stag3)	−5.744070067

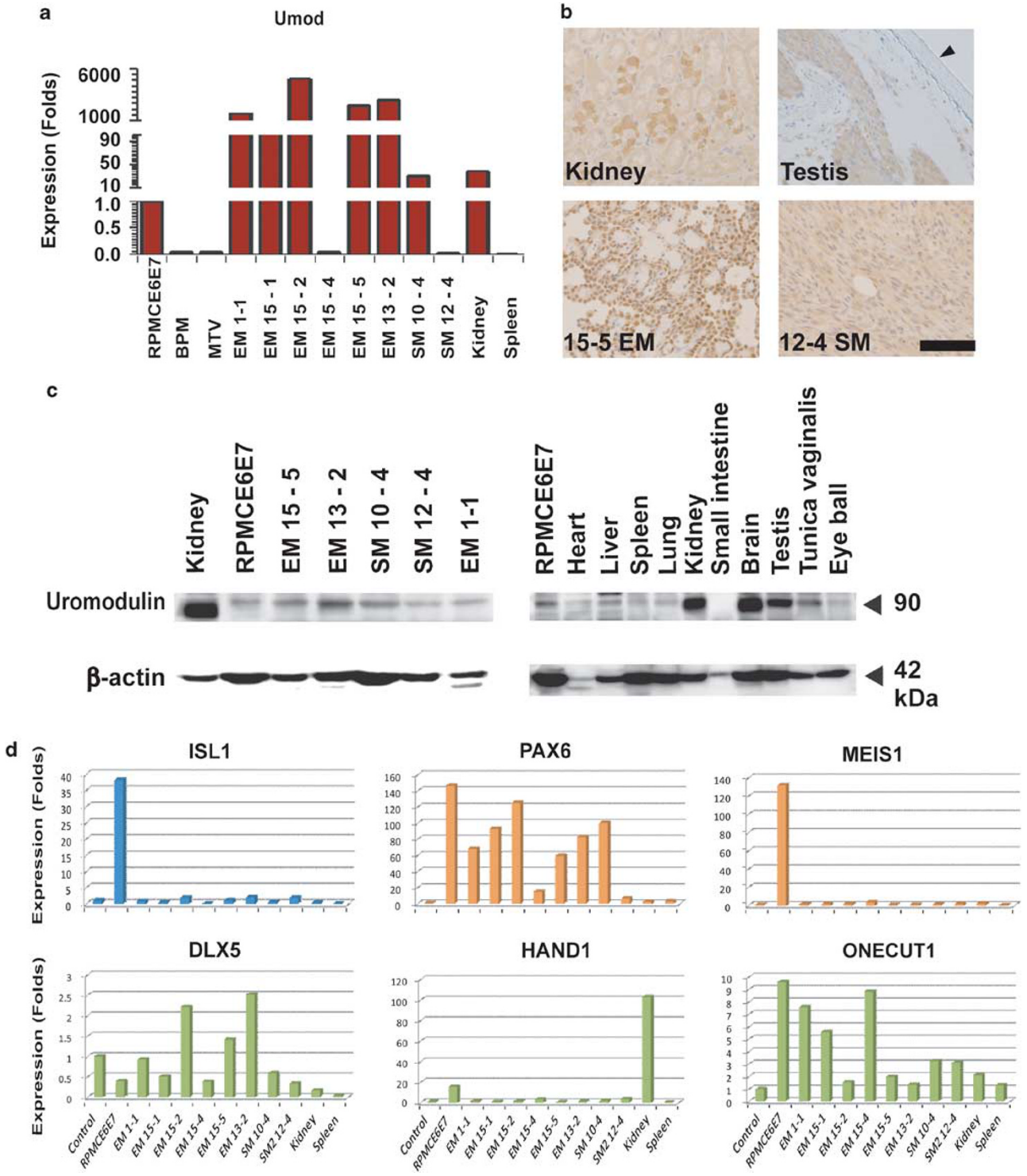


Figure 4 Expression analysis of uromodulin and mesoderm-associated transcription factors. (**a**) Levels of uromodulin message in iron-induced mesothelioma. RPMCE6E7, rat peritoneal mesothelial cells expressing E6 and E7; BPM, brushed pleural/peritoneal cells (rat); MTV, mesothelial cells surrounding tunica vaginalis testis. (**b**) Immunohistochemical analysis of uromodulin. Renal tubular cells in the ascending loop of Henle and seminiferous tubules in testis are well immunostained (Control organs are from an 8-week-old male Wistar rat). Arrowhead shows immunostaining-negative mesothelium (bar = 100 μm in kidney and testis; 200 μm in mesothelioma). (**c**) Western blot analysis of uromodulin. (**d**) Expression analysis of various embryonal transcriptional factors. PAX6 and MEIS1, ectodermal; DLX5, HAND1 and ONECUT1, mesodermal; ISL1, ectodermal/endodermal.

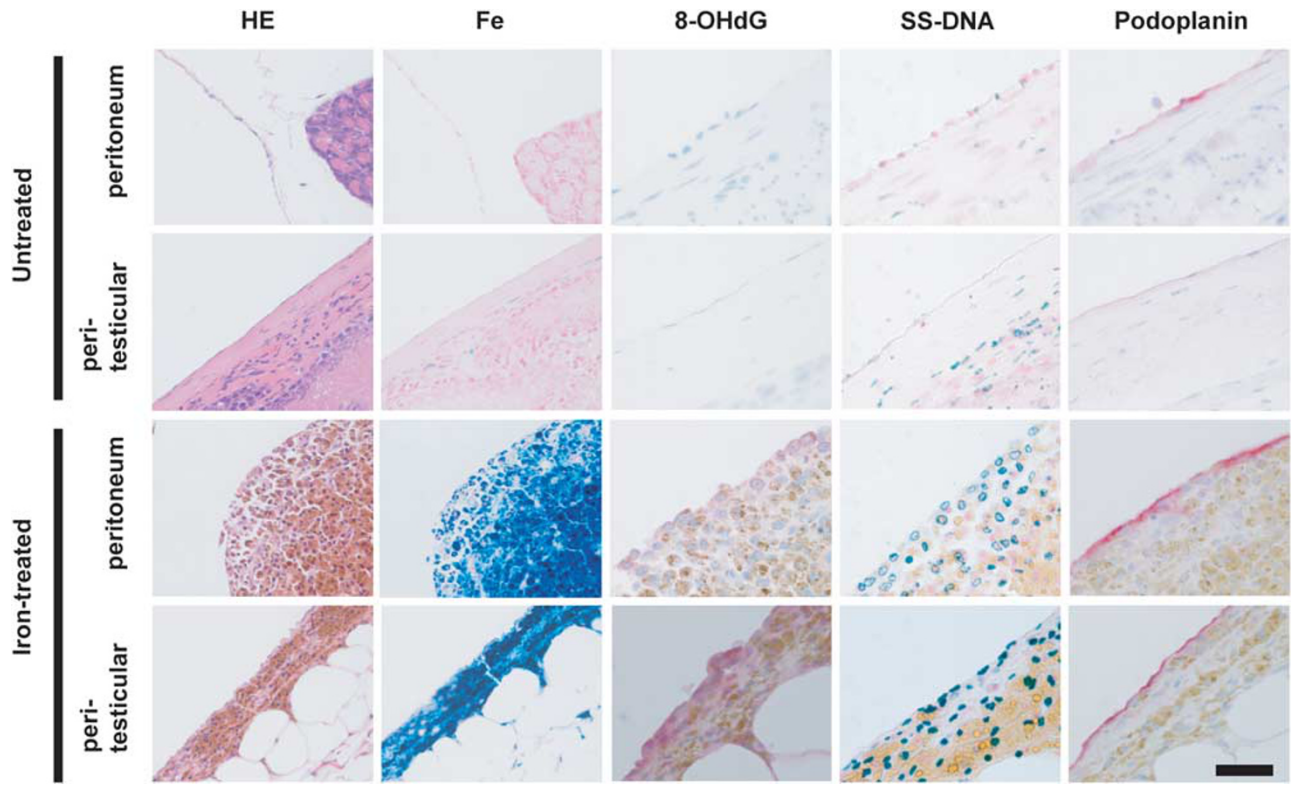


Figure 5 Analysis of oxidative stress in mesodermal cells 4 weeks after repeated iron saccharate administration. After iron-treatment, hemosiderin deposition in mesothelia and macrophages is prominent in subperitoneal areas, as demonstrated by Perls' iron staining (shown in green color). Nuclear 8-hydroxy-2′deoxyguanosine (8-OHdG) (shown in red color) and DNA single-strand breaks (shown in blue color) are increased in mesothelia after iron-treatment with immunohistochemical analyses. Surface swollen cells show podoplanin-positivity, demonstrating that they are mesothelial cells. SS-DNA, single-stranded DNA (bar = 100 μm in the right three columns; 200 μm in the left two columns).

abundant urinary protein,[15] and is immunosuppressive through interaction with interleukin 1α [59] and tumor necrosis factor by its lectin-like activity.[60] In this way, uromodulin may protect mesothelioma cells from the attack of immune cells. Regarding the discrepancy between mRNA and protein levels in the kidney and mesothelia (Figure 4a and c), we are currently studying it at the three different levels: microRNA, protein stability, and secretion.

Interestingly, we found that iron saccharate-induced mesotheliomas were driven by mesoderm-specific transcription factors, DLX5[16] and ONECUT1 (HNF6),[17] but not HAND1.[19] It is to be noted that an ectoderm-associated transcription factor, PAX6, was also activated. Thus, these molecules can be novel markers for early diagnosis and therapeutic targets if the situation is the same for human mesothelioma. Serum osteopontin (also as secreted phosphoprotein 1) levels have been proposed as a marker of mesothelioma and asbestos exposure[61–63] We could confirm this in our expression microarray profiles. Other up- or downregulated genes listed in the Tables 3 and 4 are under investigation for their roles in carcinogenesis.

In conclusion, we found two distinct pathologic types in the iron saccharate-induced mesothelioma rat model. In high-grade tumors, homozygous deletion of *CDKN2A/2B* was observed except only one case. Thus, our data strongly support that iron overload induces homozygous deletion of *CDKN2A/2B*. At the same time, iron overload is a major risk factor for the generation of mesothelioma, and any condition to induce peritoneal iron overload may eventually increase the risk for mesothelioma. Several genetically engineered mesothelioma models demonstrated the essential role of *NF2*, *CDKN2A/ARF*, and *P53* in mesothelial carcinogenesis.[64,65] However, it remains elusive how oxidative DNA damage catalyzed by iron leads to specific homozygous deletion of *CDKN2A/2B*. In this sense, this model is appropriate for the further study of mesothelial carcinogenesis and its preventive intervention.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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