Tumorigenesis and Neoplastic Progression

Chimerism of Metanephric Adenoma but Not of Carcinoma in Kidney Transplants

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Recipient-derived cells integrate into renal allografts inducing organ-specific microchimerism. Circulating pluripotent progenitor cells with high plasticity for differentiation were suggested as a potential source of allograft chimerism. Whether or not these cells also contribute to tumor formation in renal transplants is unknown. We analyzed six histologically different tumors in renal allografts for the presence of recipient-derived cells. To circumvent dependency on gender mismatch, a polymerase chain reaction assay for highly polymorphic short tandem repeat marker (DNA fingerprinting) in combination with laser microdissection was applied. Pure tumor cell populations were harvested by laser microdissection after immunohistochemical (CD45/CD68) marking of contaminating leukocytes. In cases of gender mismatch ($n = 2$), results were confirmed by sex chromosome *in situ* hybridization. Two metanephric adenomas demonstrated microchimerism comprising both donor- and recipient-derived tumor cells. Two clear cell carcinomas, one transitional cell carcinoma, and one renal cortical adenoma were all of donor origin without chimerism. We conclude that except for metanephric adenomas, tumors arising in renal transplants originate completely from graft cells. The mixed derivation of metanephric adenomas indicates an incorporation of recipient-derived progenitor cells. This finding suggests that adult stem cells can assume neoplastic phenotypes. (*Am J Pathol* 2004, 165:2079–2085)

ried recipient progenitor cells appear to be incorporated into the transplant. These stem cells display plasticity and can adopt various morphological and functional phenotypes in different types of allografts. Examples include renal tubular epithelial cells, endothelial cells, interstitial myofibroblast, hepatocytes, bile duct epithelial cells, cardiomyocytes, pneumocytes, and bronchial epithelial cells.^{1–11} Chimerism in organ transplantation is not only generated by recipient-derived stem cells, but also can originate from a spread of graft cells beyond the transplanted organ. Mazal and colleagues¹² showed that donor-derived tubular epithelial cells from renal transplants are the source for nephrogenic adenomas arising in the urinary bladder of allograft recipients. Because neoplasms arise *de novo* in solid allografts, we investigated six tumors in renal transplants for the presence of recipient-derived cells. We combined laser-microdissection of pure tumor cell populations with genotyping (DNA fingerprinting).^{5–8} This approach enabled analysis of all allografts independent of gender mismatch. In two cases, in which the recipient and donor were of different gender, we detected sex chromosomes by *in situ* hybridization to confirm short tandem repeat (STR)-polymerase chain reaction (PCR) results.

Materials and Methods

Cases

Six tumors arising in renal allografts were investigated. Five neoplasms were retrieved from the archives of the Institute for Pathology of the Medical School, Hannover, Germany. One additional case came from the Pathology Department of the University of Basel, Basel, Switzerland (Table 1). Four allografts were explanted because of

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Microchimerism after organ transplantation has been previously described.^{1–9} Pluripotent bone marrow-de-

Table 1. Clinicopathological Characteristics of Investigated Cases

Case no.	Histopathological diagnosis in allograft	pTNM and grading	Tumor diagnosis after TX (months)	Native kidney disease	Sex donor	Sex recipient
1	Metanephric adenoma	Benign	120	Congenital nephrotic syndrome	Male	Female
2	Metanephric adenoma	Benign	141	IgA nephropathy	Male	Female
3	Clear cell carcinoma	pT1pNxMx G2	93	IgA nephropathy	Male	Male
4	Clear cell carcinoma	pT1pNxMx G1	226	Chronic GN	Male	Female
5	Transitional cell carcinoma	pT3pNxMx G2	41	Chronic GN	Female	Male
6	Cortical adenoma	Benign	1	Chronic GN	Male	Male

GN, glomerulonephritis.

chronic dysfunction and neoplasms were found incidentally during pathological examination. Two were clear cell renal carcinomas and two were metanephric adenomas. One allograft was resected with the diagnosis of a transitional cell carcinoma of the renal pelvis diagnosed during a work-up of macrohematuria. Another case with the diagnosis of a small cortical adenoma was an incidental finding at biopsy for an unexplained rise of serum creatinine 4 weeks after transplantation.

Laser Capture Microdissection and DNA Preparation

Sections (3 μ m) from formalin-fixed and paraffin-embedded specimens were mounted on glass slides coated with polyethylene foil.^{5-8,13} To exclude any contamination of the following PCR analysis by infiltrating recipient cells, an immunohistochemical stain for leukocytes and histiocytes combining antibodies against CD45 and CD68 (both DAKO, Hamburg, Germany) was performed following a standard ABC technique.⁸ Laser-based microdissection of pure epithelial tumor cell populations were performed using the PALM Laser MicroBeam system (P.A.L.M., Bernried, Germany).¹³ Approximately 700 to 1000 tumor cells were dissected from each neoplasm. Only epithelial cells were harvested, leaving out stroma cells and blood vessels. After collecting the cells into the lid of a 0.5-ml reaction tube, the DNA was isolated by applying 25 μ l of proteinase K digestion buffer (50 mmol/L Tris, pH 8.1, 1 mmol/L ethylenediaminetetraacetic acid, 0.5% Tween 20, 40 μ g/ml proteinase K) into the lid. The reaction tubes were incubated overnight at 55°C, and after denaturation at 94°C for 8 minutes the lysate was used for subsequent PCR analysis.

STR-PCR

For detection of recipient-derived cells within the microdissected tumor cells, a PCR assay was applied that analyzes a highly polymorphic STR marker located within the human β -actin-related pseudogene, H- β -Ac-psi2, on chromosome 5. This marker is also known as SE33 and contains a tetranucleotide repeat that displays considerable polymorphism and a high heterozygosity rate of up to 95%.^{14,15} To increase sensitivity for partially degraded

DNA isolated from formalin-fixed and paraffin-embedded tissue, we used modified primers that reduce the length of the PCR products to fragments between 140 and 236 bp.⁷ The amplification reaction was performed in a final volume of 25 μ l containing 22 nmol/L of each primer, 0.5 U Hot Start *Taq* polymerase (Qiagen, Hilden, Germany), 1.5 mmol/L MgCl₂, 250 nmol/L of dNTP, and up to 10 μ l of DNA lysate. The reaction mixture was preheated at 95°C for 15 minutes, followed by 35 cycles at 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 1 minute, with a final elongation step at 72°C for 10 minutes. The PCR products were analyzed using a capillary electrophoresis device (ABI310; Applied Biosystems, Darmstadt, Germany). One μ l of the PCR product was mixed with 0.5 μ l of size standard (GeneScan350, Applied Biosystems) and 12 μ l of formamide. This mixture was heated for 2 minutes at 91°C and then immediately chilled on ice. Electrophoresis was done following the manufacturer's instructions. Electropherograms displaying size and intensity of the PCR products were created using the software package supplied with the instrument (GeneScan310 analysis).

To identify recipient-derived cells within the microdissected tumor cells, first the allelotype of the recipient had to be revealed. This was achieved by analogously analyzing a tissue specimen from the recipient, which had been archived before renal transplantation, or peripheral blood samples of the recipients, respectively. Analyzing the whole allograft/tumor specimen without microdissection (total DNA extraction) supplied a mixture of recipient (infiltrating cells) and donor (allograft-derived) allelotype. By subtracting the already known recipient allelotype from the mixed allelotype, the donor-specific alleles could readily be identified.

In Situ Hybridization and Immunohistochemistry

In two cases with gender mismatch transplantation additionally sex chromosome *in situ* hybridization was done to confirm STR-PCR results. For the detection of the Y and the X chromosome in the formalin-fixed and paraffin-embedded tissue, we applied a modified version of a previously described chromogenic *in situ* hybridization protocol.¹⁶ The Y and the X chromosome hybridization with specific centromere probes (Appligene-Oncor, Hei-

Table 2. Results of Chimerism Analysis by STR-PCR

Case no.	Histopathological diagnosis	Derivation	Chimerism
1	Metanephric adenoma	Donor + recipient	Yes
2	Metanephric adenoma	Donor + recipient	Yes
3	Clear cell carcinoma	Donor	No
4	Clear cell carcinoma	Donor	No
5	Transitional cell carcinoma	Donor	No
6	Cortical adenoma	Donor	No

delberg, Germany) was combined with immunohistochemistry for a pan-cytokeratin antibody (clone KL-1; Beckman-Coulter Immunotech, Krefeld, Germany) to clearly identify epithelial cells of the neoplasm. In brief, 5- μ m sections of tumor specimens were deparaffinized and rehydrated. After incubation of primary antibody the detection was done by a standard ABC technique with BCIP/NBT/INT (DAKO) as chromogen producing a blue membrane stain of epithelial cells. Subsequently a DNA probe for the centromeric region of the X or Y chromosome was incubated overnight (37°C) and detected by an optimized avidin-biotin-complex technique (NEN Life Science, Boston, MA). ACE (DAKO) served as substrate developing a red- to brown-colored nuclear dot-like signal. Furthermore, the X and the Y probe were applied simultaneously with the fluorescence *in situ* hybridization technique. Slides were pretreated as described above added by a microwave-heating step for epitope retrieval. Hybridization followed using directly labeled centromere-specific probes for chromosomes X and Y (Abbott, Wiesbaden, Germany) as described in detail earlier.¹⁷ Because renal neoplasms frequently show numerical chromosomal aberrations, we combined sex chromosome probes with a chromosome 8 probe to look for tumor tetraploidy and identify any loss of signals because of sectioning artifacts. To demonstrate clonal aberrations in cases of interest, respective chromosomes (ie, 7 and 17) were investigated for characteristic numerical aberrations by the fluorescence *in situ* hybridization technique.^{18,19}

Results

Demographic information is given in Table 1. STR-PCR after laser microdissection is shown in Table 2. Two of the investigated neoplasms, both metanephric adenomas (cases 1 and 2), revealed a chimeric genotype in the STR-PCR (Figure 1, A and B). In both cases pure populations of epithelial cells comprised cells of donor and recipient derivation. In case 1 (Figure 1A) the recipient had a homozygous allelic genotype for the SE33 locus, as described for ~5 to 7% of the general population,^{14,20} whereas the donor was heterozygous. For case 2 an inverse constellation was found with a heterozygous recipient and a homozygous donor (Figure 1B). All other

tumors, two clear cell carcinomas, one transitional cell carcinoma (both recipient and donor heterozygous for SE33 locus; Figure 1C), and one cortical adenoma, were of donor derivation without chimerism. For confirmation of STR-PCR results, additional *in situ* hybridization experiments were done (Table 3) in the two metanephric adenomas (both donor male, recipient female).

X Chromosome in Situ Hybridization

The majority of the epithelial cells within the metanephric adenomas had one nuclear X chromosome signal. In a few (<2% of epithelial tumor cells) cells, two X chromosomes were detected in the nucleus (Figure 2A). Because of the fact that 5- μ m sections were used for hybridization, only one or no sex chromosome was visible in approximately one-third of the nuclei.¹⁰ The majority of interstitially infiltrating cells, as well in the metanephric adenomas as in the adjacent allograft tissue, displayed two X chromosomes, indicating their recipient (= female) derivation. Tubular epithelial cells in the vicinity of the tumors showed mostly one X chromosome according to the male gender of the donor. Some tubular epithelial cells (<2.5%) had two X chromosomes, representing epithelial microchimerism as previously described.⁸

Y Chromosome in Situ Hybridization

All epithelial cells of both metanephric adenomas lacked the Y chromosome indicating a known chromosomal aberration for these tumors.¹⁸ Few interstitial cells between the epithelial complexes of the metanephric adenoma showed a nuclear Y signal whereas numerous cells in the surrounding fibrotic capsule and the adjacent allograft tissue displayed Y chromosomes (Figure 2B).

XY Fluorescence in Situ Hybridization

The majority of the epithelial cells of the metanephric adenomas revealed one signal for the X chromosome. A few cells had two X chromosomes and several nuclei were without any signal (Figure 2C). No epithelial cell within the metanephric adenoma displayed a XY karyotype. Adjacent tubules of the allograft had a XY constellation in numerous epithelial cells, some cells in between showed two X chromosomes, and approximately one-third of cells revealed no signal. Infiltrating cells in the interstitium were recipient-derived with two X chromosomes.

X and Chromosome 8 Fluorescence in Situ Hybridization

Within the metanephric adenomas, single epithelial cells displayed two signals for the X chromosome and simultaneously two signals for chromosome 8 (Figure 2D). Most cells showed variable constellations of one or two X chromosomes and chromosome 8 signals, but never

more than four signals in total. Again ~30% of the nuclei were without any signal.

Chromosome 7 and Chromosome 17 Fluorescence in Situ Hybridization

Both metanephric adenomas had an aberrant karyotype with monosomy 7 and trisomy 17.

Discussion

The mechanism behind the intriguing phenomenon of nonleukocyte intraorgan microchimerism is still not entirely clear. Currently, most groups favor a transdifferentiation of recipient-derived pluripotent bone marrow cells into allograft-specific cells.²¹ Some investigators presented data from animal models indicating that fusion of pluripotent stem cells with respective organ cells leads to microchimerism.^{22–25} High expectations have been placed on stem cells as an inexhaustible source for therapeutic tissue regeneration. This hope is threatened by potential side effects of pluripotent stem cells. The most well known are graft-versus-host-disease and tumor induction, as shown in animal models.^{26–29} We were able to demonstrate epithelial microchimerism in benign neoplasms arising in renal allografts. This finding suggests that recipient-derived progenitor cells might contribute to tumor formation in humans. Microchimerism was detected within two metanephric adenomas by STR-PCR (DNA fingerprinting), a highly sensitive method. The reliability has been well documented in previous studies.^{5–8,30–32} A potential obstacle to the analysis of epithelial microchimerism is the detection of pseudo-chimerism caused by a contamination with infiltrating recipient-derived inflammatory cells (eg, underlying lymphocytes) or cell fragments in the three-dimensional setting of a tissue section. We have circumvented this pitfall by combining immunohistochemistry and laser microdissection. Fragments of laser-destroyed cells do not cause contamination of STR-PCR as shown previously.^{5,6} Chimerism-negative tumors could be taken as controls that indicate elimination of the contamination problem by the methodological approach applied. Furthermore, we confirmed our STR-PCR results by X chromosome *in situ* hybridization revealing tumor cells with a XX karyotype in allografts

Figure 1. STR-PCR detecting microchimerism in metanephric adenomas (A, case 1; B, case 2; C, case 5). **A: A**, Homozygote alleleotype of recipient, determined on tissue taken before transplantation. **A: B**, Chimeric alleleotype in epithelial cells of the metanephric adenoma (red arrow is pointing to allele of homozygote recipient, black arrow is pointing to alleles of heterozygote donor). Corresponding individual peak size is given in the table at the bottom. **B: A**, Homozygote alleleotype of recipient, determined on tissue taken before transplantation. **B: B**, Chimeric alleleotype in epithelial cells of the metanephric adenoma (red arrow is pointing to alleles of heterozygote recipient, black arrow is pointing to allele of homozygote donor). Corresponding individual peak size is given in the table at the bottom. **C: A**, Heterozygote alleleotype of recipient, determined on tissue taken before transplantation (red arrows). **C: B**, Heterozygote alleleotype of epithelial cells of the transitional cell carcinoma (black arrows). Corresponding individual peak size is given in the table at the bottom.

Table 3. Results of *in Situ* Hybridization in Metanephric Adenomas

Chromosome probe	Case no. 1 donor male: recipient female	Case no. 2 donor male: recipient female
Chromosome X	TC: majority X, few XX IC: majority X AK: majority X, few XX	TC: majority X, few XX IC: majority X AK: majority X, few XX
Chromosome Y	TC: no Y IC: majority Y AK: majority Y	TC: no Y IC: majority Y AK: majority Y
Chromosome X + Y	TC: majority X, few XX, no XY IC: majority XY AK: majority XY, few XX	TC: majority X, few XX, no XY IC: majority XY AK: majority XY, few XX
Chromosome X + 8	TC: majority X88, few XX88 IC: majority X88 AK: majority X88, few XX88	TC: majority X88, few XX88 IC: majority X88 AK: majority X88, few XX88
Chromosome 7	TC: monosomy7 IC: diploid AK: diploid	TC: monosomy7 IC: diploid AK: diploid
Chromosome 17	TC: trisomy17 IC: diploid AK: diploid	TC: trisomy17 IC: diploid AK: diploid

TC, tumor cells; IC, infiltrating recipient-derived cells in the interstitium of tumor and allograft; AK, adjacent kidney cells, ie, tubular epithelial cells.

from male donors in a female recipient. To exclude the possibility that the XX karyotype is because of aberrant tetraploidy of the neoplasm we combined the X probe with a chromosome 8 probe, a locus not known to be altered in kidney tumors. We found no polyploidization. From these findings we conclude that both metanephric adenomas arising in women receiving male allografts were of host and donor derivation. All other investigated tumors, two clear cell renal carcinomas, one transitional cell carcinoma, and one cortical adenoma, were purely donor-derived and displayed no epithelial microchimerism. Possibly, the nature of the neoplasm influences its susceptibility for integration of recipient-derived cells. All nonchimeric tumors were malignant, except the cortical adenoma, which was found incidentally in an allograft biopsy taken less than 4 weeks after transplantation. This benign tumor most probably pre-existed in the allograft. This fact further strengthens the specificity of our STR-PCR approach because this adenoma could be regarded as a negative control. For all other tumors, a *de novo* genesis in the allograft can be postulated because they developed under immunosuppression years after transplantation. However, metanephric adenomas are rare benign kidney tumors that are regarded as clonal neoplasms.^{18,33} Similar to other renal tumors metanephric adenomas frequently show chromosomal aberration at chromosome 7, 17, and sex chromosomes.¹⁸ Both tumors in this study exhibited aberrations of these chromosomes with monosomy 7, trisomy 17, and loss of Y chromosome. Chromosomal aberrations suggest clonality of these neoplasms. On the basis of their aberrant karyotype, metanephric adenomas were related to papillary renal tumors (adenomas and carcinomas).^{18,34} Others did not find any chromosomal aberrations in metanephric adenoma and suggested that these clinically and morphological benign tumors might represent an unrelated and unique entity.¹⁹ Earlier reports classified these rare neoplasms as a hamartomatous element of nephro-

blastomatosis with an unusual degree of cell maturation and differentiation. Because of their embryonic architectural and cytological appearance metanephric adenomas were frequently regarded as a benign counterpart of Wilms tumor.^{18,33,35} It might be speculated that the immaturity of metanephric epithelial cells provide a microenvironment attracting pluripotent recipient-derived progenitor cells and encourage their integration into the tumor. Further investigations are necessary to prove if this tumor entity arising in transplants can serve as a model to elucidate homing mechanisms of adult stem cells *in vivo*.

Transdifferentiation of recipient-derived progenitor cells into epithelial tumor cells as the mechanism of chimerism would implicate that the recipient cells develop the same karyotype as the donor-derived counterparts during transdifferentiation. However, we were not able to find tumor cells with a XX + trisomy 17 karyotype (data not shown). Possibly, some morphological identical recipient-derived cells with an XX and epithelial (cytokeratin-positive) phenotype without trisomy 17, were present among the donor-derived tumors cells (XdelY + trisomy 17 karyotype). Such cells of recipient derivation would explain the microchimerism in the STR-PCR analysis. Another possibility is that we found no XX trisomy 17 cells because of technical reasons. Few recipient-derived cells were present in the tumors and detecting five desired chromosomes simultaneously in one level of a histological section is very difficult.

Alternatively, chimerism might have been induced by a fusion of recipient-derived pluripotent progenitor cells with allograft cells during the initial phase of tumor development. Following the results from recent animal studies one can recognize such fused cells by a mixed donor and recipient sex chromosome karyotype, such as XXXY or XXXYY.^{24,25} We were not able to find comparable tetra- or hexaploid cells in the tumors. Methodological problems (section level) may have confounded our ef-

Figure 2. Results of *in situ* hybridization of metanephric adenomas (cases 1 and 2). **A:** X chromosome *in situ* hybridization with chromogenic visualization. A few epithelial cells of the metanephric adenoma show two nuclear signals (**pale arrows**) whereas the majority display one. Infiltrating cells in the interstitium display two signals (**dark arrows**), too. Focal dark-blue membrane and cytoplasmatic staining for pan-cytokeratin is labeling epithelial cells (**short arrow**). **B:** Y chromosome *in situ* hybridization with chromogenic visualization. A few cells in the interstitium of the metanephric adenoma and numerous in the adjacent fibrotic pseudo-capsule (**arrows**) display one nuclear signal representing donor-derived stromal cells. Epithelial cells of the metanephric adenoma lack Y chromosomes in their nuclei indicating chromosomal loss. **C:** XY fluorescence *in situ* hybridization. Few cells within the metanephric adenoma display two X chromosomes (**red dots, arrow**), whereas more cells show one red signal or no signal at all. No Y chromosome (green signal) can be appreciated in epithelial cells of the tumor, while one adjacent cell in the interstitium displays one green nuclear signal (**short arrow**). **D:** Fluorescence *in situ* hybridization for X and chromosome 8. Epithelial cells in the metanephric adenoma with two X and one or two chromosome 8 signals (red, X chromosome; green, chromosome 8) can be appreciated (**arrows**). No cells were found with a tetraploid karyotype (ie, XX8888). Original magnifications: $\times 400$ (**A, B**); $\times 630$ (**C, D**).

forts. However, the fusion hypothesis arose from studies on animals investigated within 6 months after stem cell infusion.^{24,25} We investigated the tumors a decade after transplantation. Possibly, fused donor- and recipient-derived cells were present in the initial phase of those rather slow-growing tumors. Aberrations during tumor development under immunosuppression may have led to the current karyotype with superimposed loss of chromosomes.³⁶ However, the fact that the aberrations were limited to the tumor and were not found in the surrounding normal tissue or infiltrating lymphocytes makes a superimposition unlikely.¹⁸

Finally, we cannot definitely elucidate the mechanism of microchimerism induction in metanephric adenomas arising in renal allografts from our data. Nevertheless, we were able to demonstrate a chimeric derivation of this

histogenetically still undeterminate, but benign neoplasm. Because we did not find any evidence for a fusion of recipient- and donor-derived cells, a transdifferentiation of recipient progenitor cells into epithelial cells with a morphological phenotype of tumor cells can be a potential mechanism of chimerism induction in this blastemic tumor entity. However, this interpretation would imply that adult stem cells maintain transdifferentiation plasticity in humans beyond nonneoplastic cell types.

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