Assessment of Chimerism in Epithelial Cancers in Transplanted Patients

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**Abstract**
Cancer is now the most severe complication in the long term in transplant recipients. As most solid-organ or hematopoietic stem-cell transplantations are allogeneic, chimerism studies can be performed on cancers occurring in recipients. We summarize here the different methods used to study chimerism in cancers developing in allogeneic-transplant recipients, analyze their respective advantages and report the main results obtained from these studies. Chimerism analyses of cancers in transplant recipients require methods suited to tissue samples. In the case of gender-mismatched transplantation, the XY chromosomes can be explored using fluorescent in situ hybridization on whole-tissue sections or Y-sequence-specific PCR after the laser microdissection of tumor cells. For cancers occurring after gender-matched transplantation, laser microdissection of tumor cells enables studies of microsatellite markers and high-resolution melting analysis of mitochondrial DNA on genes with marked polymorphism, provided these are different in the donor and the recipient. The results of different studies address the cancers that develop in both recipients and in transplants. The presence of chimeric cells in these two types of cancer implies an exchange of progenitor/stem-cells between transplant and recipient, and the plasticity of these progenitor/stem-cells contributes to epithelial cancers. The presence of chimeric cells in concomitant cancers and preneoplastic lesions implies that the oncogenesis of these cancers progresses through a multistep process.

**Introduction**
Organ transplantations are now common\cite{1–4} and they constitute a significant improvement in the matters of public health and the quality of life for patients. Progress in the optimization of donor/recipient immunocompatibility matching\cite{5, 6} and immunosuppression protocols\cite{7, 8} has led to a significant decrease in the number and severity of acute graft rejection episodes.

The most severe complications now occur in the chronic stage, with the emergence of cancers in the long term in transplant recipients\cite{9}. The number of these
cancers is increasing along with the increase in the numbers of transplantations performed and patients with long-term transplantation [10]. The emergence of cancer can decrease the overall prognosis of the transplantation process and, therefore, the benefit of this type of therapy for the patient [11].

Most solid-organ or hematopoietic stem-cell transplantations are allogeneic [1–3], and chimerism studies are therefore appropriate [12, 13]. After hematopoietic stem-cell transplantation, tests for chimerism are used in the follow-up of the patient, and different methods for chimerism studies in blood and tissue have been validated. When applied to the cancers developing in transplant recipients, these methods can identify the donor/recipient origin of the different cellular components of the tumor [12, 14]. When these methods are applied to preneoplastic and neoplastic lesions, they offer clues to the complex process of the oncogenesis of these tumors.

**Different Types of Cancer in Transplant Patients**

**Cancers Developing in Transplant Recipients**

Kidney and liver transplantations are the most commonly performed solid-organ transplantations. In addition, kidney transplantation is the longest-standing [15], so the number of patients with long-term kidney transplantation is large [16]. In the period 2005–2008, the United Network for Organ Sharing in the USA totaled 32,258 kidney transplants, and the Collaborative Transplant Study in Europe totaled 23,530 [16].

In kidney transplant recipients, the most common cancer occurring after transplantation is nonmelanoma skin carcinoma [17]. These tumor types are also the most frequent after liver and heart transplantation [18]. Recent studies have classified the cancers occurring in transplanted patients according to their infectious or noninfectious origin. According to this classification, of the infection-related cancers, liver malignancies, Kaposi sarcoma and EBV-related non-Hodgkin lymphoma have the highest incidence when all solid-organ transplantations are considered [19]. Among noninfection-related malignancies, lung and kidney cancer, melanoma and nonmelanoma skin cancer have the highest incidence when all solid-organ transplantations are considered [19].

The highest incidence of lung cancers occurs in lung transplant recipients, possibly because of smoking, but an increased risk of lung cancer is also associated with HIV infection, independent of the patient’s smoking history [20, 21]. Numerous similarities have been found in the incidence of cancer in HIV-AIDS patients compared to immunosuppressed transplant recipients, particularly for cancer with a known infectious cause, as in human papillomavirus-related cancer and liver cancer [22, 23].

**Cancers that Develop in the Transplants**

Cancers developing in the grafts have been reported in kidney, liver and lung transplants.

De novo tumors in renal allografts are rare and their prevalence is estimated at between 0.19 (calculated on a series of 41,806 kidney transplant recipients [24]) and 0.46% (calculated in a series of primary renal cell carcinomas occurring after kidney transplant [25]). One constant characteristic of primary cancers developing in transplanted kidneys is the predominance of the papillary renal-cell carcinoma type, whereas the clear-cell renal-cell carcinoma type is the most frequent renal cancer in the general population. A multicenter French study on cancers in kidney transplants showed an incidence of 45.6% for papillary renal cell carcinomas (vs. 10–15% in the general population), most of them being confined to an organ (T1–T2) and of a low grade (G1–G2) [24]. In a German retrospective examination of 2,001 consecutive renal transplant recipients, 1.5% renal cell carcinomas were found; 25 tumors were found in native kidneys and 5 in allografted kidneys. The rate of papillary renal cell carcinoma was 37.5% [26]. Another characteristic of the primary cancers occurring in kidney transplants is the concomitant occurrence of other lesions in the kidney allograft, e.g. angiomyolipoma [27], or often papillary adenoma proliferations [28]. Clinically, the papillary renal cell carcinomas in kidney transplants occur in patients who were younger at the start of immunosuppression, and they are aggressive despite their low-stage and low-grade classification [26]. When a tumor of the renal-cell carcinoma type occurs in a kidney transplant, it is important to demonstrate that it is not a metastasis from a renal cell carcinoma developing in the native kidneys. This can be achieved with clinical data and imaging studies [29]. In imaging diagnosis of these cancers, ultrasonography sensitivity is reduced by cystic transformation of the native kidneys after renal failure [26].

Following liver allograft transplantation, the main cause of the development of hepatocellular carcinoma (HCC) in the transplant is actually HCC recurrence, a problem that occurs in approximately 20% of patients after transplantation, despite refined selection criteria and exhaustive preoperative staging [30]. However, de novo HCC in liver grafts have also been observed in transplant recipients with no previous evidence of HCC [31–33].
These true de novo HCC can be associated with recurrent hepatitis B [34] or with sustained hepatitis C virus clearance after liver transplantation [35]. They can also be associated with cirrhosis linked to recurrent hepatitis B [36] or C. To distinguish de novo HCC from recurrent HCC, a useful clinical element is the mean time-lapse to occurrence, which is around 5 years after liver transplantation for de novo HCC as opposed to approximately 2 years for recurrent HCC [35].

Cancers developing in lung transplants are squamous cell carcinomas, adenocarcinomas or more rarely bronchoalveolar carcinomas and sarcomas. In 2 separate studies, the incidence of these bronchogenic cancers in lung transplant recipients was 2.4% in 290 transplant recipients [37] and 2.6% in 345 transplant recipients [38]. The average interval from transplantation to the development of lung cancer is 5 years. These cancers occurred in patients with a history of smoking [37, 38] and are usually diagnosed at an advanced stage and have a poor outcome [37].

Cancers Transferred with the Transplants
Cancers of donor origin in transplant recipients can be transferred with the transplant. This is a rare event. Its occurrence is evaluated at between 0.05 [39] and 0.02% [40].

Transfers of malignant melanoma and choriocarcinoma, two tumors with a high hematogen metastatic potential, have been reported with kidney transplants [41, 42]. In the UK Registry [39], renal cell carcinomas have also been found to be transmitted in kidney transplant recipients, non-small cell lung cancer in lung transplant recipients and colon adenocarcinoma and neuroendocrine tumors in liver transplant recipients. One major challenge for solid-organ transplantation teams is the approval of patients with primary brain tumor to be donors, because the extraneural spread of primary brain tumors is rare and there is a critical shortage of donors for transplantation. One study found that glioma cells were transferred in this manner in a pancreas transplant [40].

After allogeneic bone marrow transplantation, malignant diseases transferred with the transplant include leukemia [43], Hodgkin lymphoma and non-Hodgkin lymphoma [44, 45].

### Different Methods for the in situ Chimerism Study of Cancers in Transplant Patients

Table 1 shows the different methods used for chimerism studies, which are currently used in the follow-up of patients after hematopoietic stem-cell transplantation. These studies are performed on blood cells using the microsatellite markers of highly polymorphic genes. The first step is the analysis of the donor and recipient DNAs before the transplantation, using a series of microsatellite markers with short tandem repeat sequences of different lengths. Microsatellite markers are chosen only when donor and recipient DNAs before the transplantation are different.

As a second step, the selected microsatellite markers are tested at different times after transplantation in order
to monitor the number of chimeric cells (in most instances, recipient-derived) in the blood of the recipient. Decreasing values of donor chimerism are predictive of transplant failure and relapse of disease [46].

The reported average sensitivity of this method in detecting minor cell populations is not >5%, but it is highly specific and can easily be quantified in a given number of blood cells [47].

These methods using microsatellite markers can be transposed for chimerism studies of solid cancers, using in situ hybridization or PCR after the laser microdissection of tumor cells. Fluorescent in situ hybridization (FISH) for microsatellite markers can be performed on fixed- and frozen-tissue sections. Not all fluorescent probes for microsatellite markers of highly polymorphic genes are commercially available and they may have to be designed and labelled [48]. On tissue sections studied with FISH, the number of positive cells per field is easy to quantify. The type of positive cells in the tissue sections can also be identified either from morphologic criteria or by using immunostaining combined with FISH [14]. The transposition of PCR methods for blood cells to analyze cancer tissue requires the selection of tumor cells, which can be performed using laser microdissection [49]. However, a minimum number of tumor cells have to be microdissected to perform the PCR and its controls. Since laser microdissection is currently performed on 7-μm-thick tissue sections, sections of 500–1,000 tumor cells are necessary [49]. In the case of tumors associated with an extensive inflammatory reaction, it is necessary to check the quality of the laser-microdissected cell population using PCR with an epithelial marker and a pan-leukocyte marker to be sure that no inflammatory cells have been microdissected along with the tumor cells. DNA extraction from these small quantities of biological material is optimized using specific kits [50]. The microsatellite probes and PCR protocols are similar to those used for blood cells [47]. Similarly, the method of high-resolution melting (HRM) of mitochondrial DNA, currently used for filiation inquiries [51] on cells in suspension, can be transposed to chimerism studies of cancer tissue sections [52]. For this type of analysis, the molecular target is the D-loop of the mitochondrial DNA. This method requires the availability of donor and recipient DNA and a selection of tumor cells using laser microdissection of tissue sections. As for microsatellite analysis, a minimum number of microdissected tumor cells are required for the PCR and its controls. The molecular probes and PCR protocols are similar to those performed on cell suspensions.

The new droplet digital PCR (ddPCR) can enable both the identification of chimeric cells and relative counts of chimeric cells in a given number in microdissected cells. In the context of gender-mismatch transplantation, ddPCR can identify Y-bearing cells in female recipients in the same way as classic PCR. ddPCR is an emulsion-based PCR process which performs absolute quantification of nucleic acids. The first step is the division of a duplex fluorescent-probe-based PCR assay into approximately 20,000 highly uniform micelles with a volume of 1 nl. Each droplet in the emulsion is an independent nano-PCR. After PCR, the droplets are focused into a single-file beam of droplets which flows through a cytometer under LED excitation [53]. This enables enumeration of PCR-positive and PCR-negative droplets. In the context of cancer in transplant patients, ddPCR can thus identify the number of Y-bearing cells in a given number of microdissected cells.

**Results and Discussion**

The main result obtained from chimerism studies of cancers in transplant recipients is the identification of the chimeric cells within these cancers. This identification enables discussion on (1) the migration of the chimeric cells into the cancer, and particularly on what the triggering factor initiating this migration could be and (2) which type of cell is at the origin of the chimeric cells. In kidney transplant recipients, donor-derived cells were first identified in a basal cell carcinoma occurring in sun-exposed areas of the skin [12]. The method used was the detection of the Y chromosome in female recipients of male kidney transplants. Conversely, recipient-derived cells have been identified in renal cell carcinomas that developed in a kidney transplant. In two different studies [29, 54], the method used was a comparison between recipient blood and renal-cell carcinoma microsatellites to confirm the recipient origin of neoplastic cells.

The identification of chimeric cells in cancers in transplant recipients, either in the recipient’s skin or in the kidney transplant, demonstrates that a migration of stem-/progenitor cells has occurred. The triggering factor for chimeric cell migration in these cancers could be a process of inflammation and tissue repair. In nonneoplastic tissue, particularly in the microvessel network, chimeric cells have been found at repair sites. In the 3 months following kidney transplantation, donor-derived endothelial cells have been identified in the vessels of the kidney transplant, close to the surgical suture [55]. In the 100 days following bone marrow transplantation,
donor-derived cells were found in the microvessels and epidermis of patients, only if they had undergone acute graft-versus-host disease. In addition, the numbers of apoptotic epidermal or endothelial cells were significantly related to the numbers of chimeric cells in the epidermis or microvessels, thus confirming the relationship between the intensity of tissue damage and the density of chimeric cells. In solid-organ transplant recipients, no graft-versus-host disease occurs, but the transplantation process itself induces tissue damage. After human lung transplantation, chimeric cells in the pulmonary epithelium [56] as well as in the endothelium of microvessels [57] have been found to be associated with transplant rejection. The participation of recipient-derived cells in endothelial repair has also been reported in liver [58] and heart [59] transplants. Two mechanisms have been described in endothelial cell repair: the local migration and proliferation of endothelial cells adjacent to the site of injury and the homing and incorporation of endothelial progenitor cells at this site [60]. Unlike the endothelium, the epithelium can be pluristratified, but the homing and incorporation of progenitor cells at the site of injury is also observed when epithelial progenitor cells become exhausted by severe or chronic injury [61, 62].

Even if the migration of a progenitor cell is demonstrated by the identification of chimeric cells in cancers of transplant recipients, the type of progenitor cell involved has still to be established. In cancers occurring in bone marrow transplant recipients, hematopoietic stem cells are infused. These can be the origin of rare but authentic donor-derived posttransplant leukemia. Since the original description in 1971, more than 50 cases of donor-cell leukemias have been reported and considered to be the result of the oncogenic transformation of apparently normal donor hematopoietic cells in the transplant recipient [43]. The occurrence of epithelial carcinoma of donor origin after bone marrow transplantation implies a supplementary step of transdifferentiation of marrow stem cells in normal epithelial cells [13, 14, 63]. It has been shown that bone marrow cells contribute to epithelial cancers of the small bowel, colon, lung and skin [64]. The possibility that these cancers might stem from mesenchymal stem cells that are coinfused with hematopoietic stem cells within the marrow transplant cannot be excluded. In a kidney transplant recipient, we were able to demonstrate the presence of the same p53-mutation in the tumor cells of a skin squamous cell carcinoma and in epithelial tubular cells in the kidney transplant in a biopsy performed 7 years before the occurrence of the skin squamous cell carcinoma [65]. To date, this is the only observation demonstrating the contribution of donor epithelium to the epithelial cancer in the recipient.

With regard to the cancers occurring within the transplants, in grafted kidneys, the majority are papillary renal cell carcinomas [24], i.e. epithelial tumors. The presence of recipient cells in these cancers, found in a series of human de novo papillary renal cell carcinomas [54], opens up discussion about the type of recipient cells that homed to the transplant cancer. The first hypothesis is homing to metastatic cells from a papillary renal cell carcinoma developing in one of the native kidneys when these have not been removed. Another possibility is the engraftment of exfoliated normal tubular cells from the native kidneys coming into the transplanted kidney via a mechanism of vesicoureteral reflux. In kidney transplant recipients, as in normal individuals, thousands of exfoliated renal tubular epithelial cells are shed into the urine each day [66]. These are able to establish viable cultures [67, 68] and form nephrogenic adenoma [69]. Finally, the migration and homing of circulating, normal recipient-derived progenitor cells to the transplanted kidney could occur. Populations of progenitor/stem cells resident outside the organ are able to contribute to the renewal of different lineages, even in the tissue from a separate germ layer [70].

Preneoplastic lesions can occur along with cancers in transplant recipients. The intent of the chimerism studies of preneoplastic lesions and cancers both occurring in the same patient is to assess whether there is a homing of progenitor cells at the early preneoplastic stage, and to compare the number of chimeric cells in the lesion, in the cancer and in the normal skin, all in the same patient.

In kidney transplant recipients, the main type of cancer is squamous cell carcinoma occurring on sun-exposed areas of the skin [9]. These squamous cell carcinomas are multiple and are often associated with multiple actinic keratosis [71, 72]. In 2 female recipients of male-kidney transplants who had noncontiguous actinic keratosis and squamous cell carcinoma and for whom tissue samples and DNA from both donors and recipients were still available, we conducted a chimerism study using four independent methods: FISH for X and Y chromosome detection, ZFYqPCR, polymorphic microsatellite analysis and mitochondrial DNA HRM analysis [73]. p16INK4A immunohistochemistry (clone JC8, Biocare-Medical) and in situ hybridization using an INFORM HPV III Family 16 Probe (Roche-Diagnostics, Basel, Switzerland) showed the absence of oncogenic HPV expression in these squamous cell carcinomas. XY FISH combined with cytokeratin on the same tissue section showed chimeric XY cells in the basal-layer actinic keratosis and in the basal layer and in-

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vasive areas in squamous cell carcinoma. No chimeric cell was found in normal surrounding skin. Count showed a mean percentage of 4.5% of chimeric cells in the squamous cell carcinoma studied and 2% in the actinic keratosis studied. Laser microdissection, performed on tissue sections after immunostaining inflammatory cells, enabled us to collect only keratinocytes from the basal layer of the actinic keratinosis and keratinocytes from the basal layer and invasive areas in the dermis of the squamous cell carcinoma. The polymorphic microsatellite marker analyses and mitochondrial DNA HRM performed on the laser-microdissected cells also showed the presence of cells of donor origin in the squamous cell carcinoma and actinic keratosis. The distribution of chimeric cells in the basal layer and invasive areas of the squamous cell carcinoma corresponded to the ‘outer proliferating layer’ where tumor-initiating cells were recently characterized in skin squamous cell carcinoma [74]. Chimeric cells were also found in the basal layer of actinic keratosis, a disease usually considered as benign, although molecular studies have shown a frequent loss of heterozygosity [75]. The presence of chimeric cells in actinic keratosis and squamous cell carcinoma, and not in the normal surrounding skin, could be linked to the remodeling of tissue accompanying disease progression, as progenitor cells are recruited to the sites of skin injuries in experimental conditions [76] and tissue repair mechanisms in common with stem-cell renewal in carcinogenesis [77]. The detection of chimeric cells at the stage of actinic keratosis is in favor of a multistep process in the progression from actinic keratosis to squamous cell carcinoma.

Regarding cancer in transplant recipients that has developed in the transplant, papillary renal cell carcinoma has a high incidence in kidney allografts [24]. It is frequently multifocal [78] and is associated with papillary adenoma, an epithelial lesion considered as benign and morphologically indistinguishable from papillary renal cell carcinoma, except by its size (<5 mm) [79].

As for actinic keratosis and squamous cell carcinoma occurring in the skin of kidney transplant recipients, the concomitant development of multiple tumors and the papillary adenoma-carcinoma association make papillary renal cell carcinoma an interesting model to gain insight into the evolutionary process of human cancer. Two models have been proposed: either cancer progenitor cells drive tumor initiation and progression (cancer stem-cell model) or over time, a premalignant or malignant cell population acquires multiple mutations, genetic instability and uncontrolled proliferation (clonal evolution model) [80–82].

In 5 kidney transplant recipients with de novo papillary renal cell carcinoma and papillary adenomas in kidney transplants, XY FISH, polymorphic microsatellite DNA and mitochondrial DNA HRM analyses on laser-microdissected tumor cells identified recipient-derived cells in both papillary cell carcinoma and papillary adenoma. Counts were performed on XY FISH sections. The proportion of tumor cells with recipient chimeric cell characteristics was 90.2–94.5% in papillary carcinomas and 94.1–98.8% in the papillary adenoma. We did not find tumor cells with donor characteristics in these recipient-derived papillary renal tumors [54]. These data demonstrated an identical origin for the precursor cells in the two lesions, which were located in the same original microenvironment and had the same cellular aspect, and thus had the same degree of cellular differentiation. There were differences in size, the benign tumors being smaller than the malignant ones, and in molecular characteristics, the malignant tumors having additional genetic alterations. All these elements are in favor of a multistep molecular process [80, 81] leading to papillary renal cell carcinoma.

In conclusion, chimerism assessment in epithelial cancers in transplanted patients identified a homing of progenitor cells of donor origin to recipient cancers and, conversely, a homing of progenitor cells of recipient origin to transplant cancers. It also showed a homing of these cells to associated premalignant lesions, a fact in favor of successive steps in the carcinogenesis of these epithelial cancers.

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