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ORIGINAL ARTICLE

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Successful xenografting of cryopreserved primary pancreatic cancers

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Abstract In order to assess the suitability of cryopreserved neoplastic tissues for xenografting into nude (nu/nu) mice, we compared the take rate in 28 samples of pancreatic ductal carcinoma. Eleven fresh samples were implanted in nu/nu mice, and 17 were frozen in cryopreserving solution and implanted at a later time. All samples were examined for the presence of neoplastic tissue in cryostat sections. A total of 15 tumors grew in the animals; five from the freshly implanted samples and ten from those cryopreserved. Ten xenografted tumors were characterized for alterations in *p53*, K-*ras*, and *p16* genes, which were found in six, eight, and nine cases, respectively. Our results demonstrate that the take rate for xenografting is comparable between cryopreserved and fresh tissue samples. The procedure allows for the exchange of tumor material between institutions and permits the establishment of centralized facilities for the storage of an array of different primary tumor samples suitable for the production of in vivo models of cancers.

Keywords Pancreas · Ductal carcinoma · Xenograft · Tissue bank · K-*ras* · *P16* · *P53*

Introduction

The identification of genetic and biochemical alterations in pancreatic cancer is providing clues for the understanding of its pathogenesis and has the potential to provide new tools to assess prognosis and find novel therapeutic targets. The availability of cellular models is fundamental for these goals, because the major problem when dealing with ductal carcinoma of the pancreas is the paucity of the neoplastic cells, often embedded within a strong desmoplastic reaction. Sophisticated enrichment techniques, including cryostat enrichment and microscope-driven dissection, have been used to obtain a suitable cancer cellularity from primary cancers and their possible precursors for genetic studies [1, 10, 19]. However, the amount of tumor material obtained is limited, and these procedures can also introduce a bias in the selection of samples. Cryostat enrichment is only possible in the 20% of pancreatic cancers with a primarily high cellularity [1]. Microdissection allows the characterization of a selected sample of cells, which may be not representative of the tumor bulk.

Much research in pancreatic carcinoma has been performed using a relatively limited number of cell lines [13]. While these may represent a good starting point for investigation, this model suffers from several disadvantages. In most cases, reference DNA is not available, and numerous culture passages may introduce a bias in the significance of the analysis performed. For example, there have been reports suggesting a higher mutational frequency in cell lines with respect to primary cancers [2, 8, 20].

Xenografting human pancreatic cancer in nude (nu/nu) mice provides an excellent alternative for several reasons [7, 9, 15]. The success of the implant is much higher than the establishment of cell lines from primary tumors, thus allowing for the establishment of a wider library of neoplastic phenotypes, growing in conditions that more closely resemble the original one. In addition, once established, xenografted tumors may be propagated through implantation into additional mice, showing a high degree of genetic stability during subsequent passages [14] and have been utilized for preclinical studies on drug effects [11, 12]. Finally, the ability of neoplastic clones to establish local or distant metastases associated with specific genetic alterations [14] in nu/nu mice allows for the recognition and isolation of selected subclones that would be impossible to recognize in vitro.

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However, the organization, cost, and facilities necessary to obtain such material are beyond the reach of many laboratories due to the complex organization required. The procedure must be performed under sterile conditions, the fresh tissue must be coordinately handled by different operators, and the whole procedure must be completed within a few hours to assure the viability of the cells in the sample.

We report that xenografting of cryopreserved specimens from primary pancreatic cancers is as successful as the implant of fresh samples. The procedure described here greatly simplifies the complex organization required for xenografting and represents a major improvement in the field. In fact, while successful reimplantation of cryopreserved xenografted tumors has been reported [5, 7], the implant of cryopreserved intact specimens from primary tumors has been reported as unsuccessful [5].

Materials and methods

Materials

RPMI 1640, fetal bovine serum (FBS), and glutamine were purchased from Bio-Whittaker, Bergamo, Italy. Gentamicin sulphate was purchased from Schering-Plough, Milan, Italy, and fungizone, xylazine, ketamine, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich, Milan, Italy.

Animals

nu/nu Swiss mice (4–5 weeks old), weighing 18–22 g (Charles River, Milan, Italy), were used for tumor implantation and propagation. Animals were housed in a sterile environment; cages and water were autoclaved. Both bedding and food were γ-ray sterilized. The animal research complied with national legislation and with the GlaxoWellcome company policy on the care and use of animals and with related codes of practice.

Human pancreatic carcinoma implantation and perpetuation

Fresh surgical specimens of 28 human pancreatic ductal carcinoma were used. Cryostat sections of different fragments were analyzed to verify the presence of neoplastic cells. The specular half of the chosen sample was placed in cold transfer medium (Leibovitz L-15, BioWhittaker, Bergamo, Italy) and transferred to the laboratory where it was reduced to 1–2 mm^3 fragments under sterile conditions. Aliquots (10–15 fragments each) of the primary tumor fragments were frozen at 1°C/min using a Nalgene cryo 1 freezing container (Nalgene, Milan, Italy) in a freezing solution composed of 10% DMSO, 30% FBS, and 60% RPMI 1640 for a minimum of 4 h and then transferred to liquid nitrogen. Before transfer to the animal facility located at Glaxo-Wellcome laboratories, 11 fresh tissues and 17 vitally frozen samples were soaked into matrigel for at least 20 min. The cryopreserved tissues were quickly thawed at 37°C and washed twice in RPMI 1640 before being soaked into matrigel. A minimum of three mice were implanted subcutaneously with 2–5 tissue fragments. Tumor formation was checked weekly by visual inspection and palpation. Mice were sacrificed when the tumor reached at least 1 cm in diameter. The tumor was removed and divided into three fragments. The first was frozen using liquid nitrogen and stored, the second was

Table 1 Pancreatic ductal carcinomas implanted in nu/nu mice

Case	Gender/age (years)	Tumor location	Differentiation	Sample	Number of mice implanted	Number of mice with tumor growth	Time of growth (months)
PDX1	Female/69	Head-body	Moderate	Fresh	5	2	5
PDX2	Female/51	Head	Moderate	Fresh	5	4	2
PDX3	Male/59	Head	Poor	Fresh	5	2	2
PDX4	Female/52	Head	Moderate	Fresh	3	2	3
PDX5	Female/75	Head	Moderate	Fresh	4	4	2
PDX6	Male/57	Head	Poor	Cryopreserved	5	2	4
PDX7	Male/55	Head	Poor	Cryopreserved	4	2	3
PDX8	Female/66	Head	Poor	Cryopreserved	4	2	5
PDX9	Male/51	Head	Moderate	Cryopreserved	3	2	3
PDX10	Male/61	Head	Moderate	Cryopreserved	3	1	4
PDX11	Female/62	Body-tail	Moderate	Cryopreserved	5	2	4
PDX12	Female/62	Head	Poor	Cryopreserved	5	1	1
PDX13	Male/57	Head	Moderate	Cryopreserved	5	1	4
PDX14	Female/44	Head	Moderate	Cryopreserved	5	1	4
PDX15	Male/69	Head	Anaplastic	Cryopreserved	5	1	2
DC1	Male/77	Head	Poor	Fresh	5	None	–
DC2	Male/72	Body	Moderate	Fresh	3	None	–
DC3	Female/66	Head	Moderate	Fresh	3	None	–
DC4	Female/76	Head	Moderate	Fresh	3	None	–
DC5	Male/69	Head	Poor	Fresh	3	None	–
DC6	Male/51	Head	Moderate	Fresh	4	None	–
DC7	Male/69	Head	Poor	Cryopreserved	3	None	–
DC8	Male/42	Head	Poor	Cryopreserved	5	None	–
DC9	Female/58	Body-tail	Well	Cryopreserved	3	None	–
DC10	Male/79	Head	Moderate	Cryopreserved	2	None	–
DC11	Female/75	Head	Moderate	Cryopreserved	5	None	–
DC12	Female/55	Head	Moderate	Cryopreserved	5	None	–
DC13	Male/71	Body-tail	Moderate	Cryopreserved	5	None	–

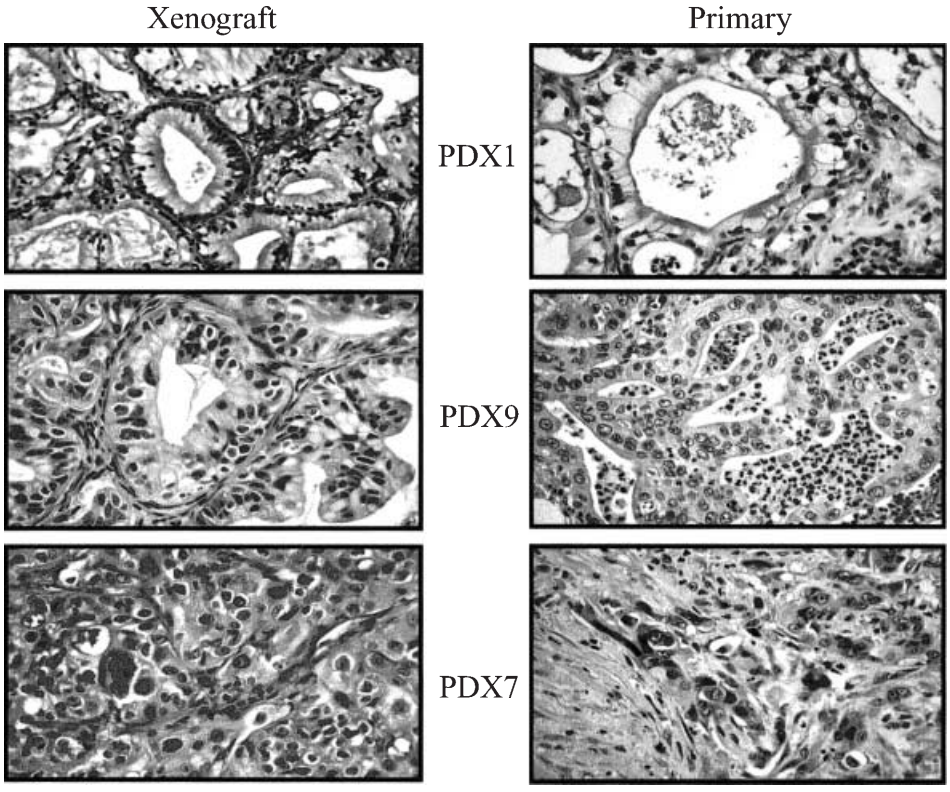

Fig. 1 Comparison of morphology between xenografts and primary tumors

Table 2 Alterations of K-*ras*, *p53*, and *p16* in pancreatic ductal cancer xenografts. *HD* homozygous deletion

	K-*ras*		*p53*		*p16*	
	Alteration	Predicted product	Alteration	Predicted product	Alteration	Predicted product
PDX1	12 GGT-GTT	Gly to Val	273 CGT-CAT	Arg to his	HD	Absent
PDX2	12 GGT-GAT	Gly to asp	None found	Wt	HD	Absent
PDX3	12 GGT-GTT	Gly to val	248 CGG-CAG	Arg to gln	94 CTG-CGG	Leu to arg
PDX4	12 GGT-GAT	Gly to asp	None found	Wt	77 ACT-A	Frameshift
PDX5	12 GGT-GAT	Gly to asp	214 CAT-CGT	His to arg	HD	Absent
PDX6	12 GGT-CGT	Gly to arg	273 CGT-CTT	Arg to leu	Methylated	Absent
PDX7	None found	Wt	None found	Wt	110 TGG-TAG	Trp to stop
PDX8	None found	Wt	None found	Wt	None found	Wt
PDX9	12 GGT-GTT	Gly to val	175 CGC-CAC	Arg to his	HD	Absent
PDX10	12 GGT-CGT	Gly to arg	282 CGG-TGG	Arg to trp	55, 10 bp ins	Frameshift

reduced into small fragments before being transferred into cryopreserving solution, and the remaining fragment was fixed in formalin for histological analysis. In addition, at least one fragment was incubated in collagenase type II (Biochrom KG, Berlin, Germany) for a few hours for tissue dissociation, washed, and plated in six-well cell culture dishes (Beckton-Dickinson, Milan, Italy) after resuspension in RPMI 1640 supplemented with 10%FBS and antibiotics. Each neoplasm was subsequently reimplanted in new sets of mice for 2–5 passages.

Mutational and methylation analysis

Polymerase chain reaction (PCR) amplified products were sequenced directly on an ABI Prism 377 instrument. Typical reactions were normally performed in a volume of 10 µl using 40 ng DNA with 35 cycles. Primers for amplification of exons 1–2 of the *p16* gene [18], exons 5–8 of the *p53* gene [16], and K-*ras* exon 1 [17] were as described. Methylation-specific PCR for the 5′ CpG island of the *p16* gene was carried out as described [18].

Results

Tumor implantation

Table 1 summarizes the clinicopathological features and the results of xenografting of the 28 ductal carcinomas implanted in nu/nu mice. The overall success rate was 53% (15/28). Of the 17 implants of cryopreserved tissue and the 11 fresh tissue fragments, 10 (59%) and 5 (45%), respectively, led to tumor growth. In particular, the tumor developed in 15 of 72 (21%) mice implanted with

cryopreserved tissue and in 14 of 43 (33%) implanted with fresh material.

Histological features of the xenografted tumors

Figure 1 exemplifies the morphological features of the tumors grown in nu/nu mice. It is evident that the xenografted sample resembles the original tumor.

Alterations in the *p16*, K-*ras*, and *p53* genes

Alteration in K-*ras*, *p16*, and *p53* are among the most frequent events observed in pancreatic ductal cancers. Therefore, ten xenografts, whose DNA was available at the time of writing, were analyzed for aberrations in these genes. The results are summarized in Table 2. For the *p16* and *p53* genes, sequence analysis detected only the mutated allele, indicating a loss of the normal allele.

Discussion

Our results demonstrate that: (1) it is possible to cryopreserve human pancreatic tumor fragments without enzymatic dissociation and obtain successful implantation in nu/nu mice and (2) there is no advantage in using fresh tissue instead of vitally frozen specimens for xenografting in nu/nu mice.

Cryopreservation of tissues is used routinely for transplant purposes. However, its use for creating a vital bank of cancer tissues has not been reported. It has been reported that the frequency of successful tumor take in nu/nu mice ranges from 50% to 80% when using fresh primary pancreatic cancer tissues [3, 5, 6, 7, 14], while the only attempt to use cryopreserved primary tumors reported in the literature had been unsuccessful [5]. In this latter report, the authors concluded that, although the procedure of vital freezing for intact tumor specimens is possible, its implementation for primary tumor samples require technical improvements.

We were interested both in establishing a pancreatic ductal carcinoma tissue bank growing in nu/nu mice and in reducing the extent of coordination necessary to implant fresh samples. Therefore, we processed fresh neoplastic fragments and compared the yield of these implants with those obtained using cryopreserved tissue. We obtained comparable success rates using vitally frozen or fresh tissue implants. There was no significant correlation between any of the variables reported in Table 1, including grade and the success of the implant.

According to our experience, the success of the entire procedure is dependent upon: (1) immediate soaking of the sample in a suitable “transfer” solution kept at 4°C, where it can be also stored for a few hours; (2) controlled 1°C/min freezing after reduction in a sterile environment; (3) quick thawing of the sample at 37°C followed by washing in culture medium before transfer into matrigel, where it should be soaked for at least 20 min; and (4) implantation of more than one fragment for each mouse.

The availability of vitally frozen primary samples offers the advantage of scheduling implants in nu/nu mice according to the availability of animals and/or operators, thus allowing relevant time and cost savings. Cryopreserved tissues can also be prepared and stored in any center and delivered at a later time to a xenografting facility. Additionally, once the first tumor growth has been obtained, cryopreservation provides a safety reservoir in case of failure of reimplants or mouse death. This is demonstrated by case PDX7; the first reimplant of freshly isolated xenograft fragments failed, but a second implant in a new set of mice using vitally frozen fragments was successful. Furthermore, a frozen bank of vital xenografts allows for the storage of a representative series of different cancer genophenotypes that can be expanded whenever necessary, thus overcoming the problem of a shortage of biological material or the necessity to increase the propagation-related costs and efforts prohibitively.

Progress in the application of molecular diagnostic techniques is providing information regarding the genetic alterations of neoplastic cell and tissue samples. It is likely that in the near future, this information will be exploited at the clinical level. In the meantime, for applications, such as the evaluation of drug response, the knowledge of the inactivation of a particular gene or molecular pathway of a cellular model is becoming increasingly important for both cell lines and animal models of disease [4]. The use of tissue vital cryopreservation would also allow for the collection of cancer samples from centers that do not have a nu/nu mice facility and wish to contribute with biological material that can be shipped at a later time. This cryopreserved pancreatic cancer tissue bank, initially characterized for alteration in the *p53*, *p16*, and K-*ras* genes, might allow for the selection of the most appropriate model for current and future experimental applications.

Although the gold standard remains to be primary cancer tissue, this is often available only in limited amounts or as paraffin-embedded formalin-fixed material, which is inadequate for extensive molecular analysis. Obviously, this material is the only one permitting validation of anomalies identified through studies on cell lines and/or xenografts. Even if miniaturization and array technology are paving the way to single-cell analysis, xenografts can be appreciated and exploited for diverse and specific applications.

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