Pathol. Oncol. Res. (2012) 18:663–668
DOI 10.1007/s12253-011-9492-6

RESEARCH

A Proposed New Technique in Prostate Cancer Tissue Bio-Banking: Our Experience with a New Protocol

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Received: 12 December 2010 / Accepted: 19 December 2011 / Published online: 4 January 2012

Abstract The aim of our study, beyond validating a method of collecting and storing biological samples from patients with prostate cancer, was to validate an innovative biopsy method for the creation of a biobank of prostatic frozen tissues. Patients referred to our hospital between November 2008 and March 2010 to undergo radical prostatectomy were invited to participate in the study. Each patient's data were stored in two databases (personal information and clinical database) while samples of urine, blood and its derivatives, fresh material and formalin-processed tissue were stored in a correlated biobank. The proposed method for collecting fresh material was to take samples of the neoplastic tissue by carrying out targeted biopsies in the area indicated by the biopsy mapping as the site of the malignancy, under manual palpation to identify the neoplastic nodule. The site of sampling was marked by an injection of India ink. 55 patients agreed to participate in the study. In 43 cases biopsies were correct, with a mean of 48% of core involved by tumour (range, 10–90%). Overall the tumour detection rate was 78.2%. The protocol for collecting biological material and the new method for collecting fresh tissue reduce internal steps and staff involved, thereby reducing all those variables that cause heterogeneity of material and changes in its quality. This process provides high quality, low cost material for research on prostate cancer. The features of the collection protocol mean that the protocol can also be used in non-academic centres with only limited research funds.

Keywords Prostate · Cancer · Prostate cancer · Bio-bank · Tissue · Biopsy

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Introduction

Biobanks of tumour tissue and biological fluids are of considerable importance in the field of preclinical and clinical research. The possibility for research groups to access such banks of samples and databases is becoming of ever greater interest, such as to require simplification and international standardisation of the procedures used.

Research related to prostate cancer is hampered by the limited availability of biological samples with associated clinical and follow-up data. This is related to the fact that few centres have the infrastructure and staff specifically dedicated to the creation of a network for the management of these processes and to the difficulty that the numerous non-academic centres have in obtaining sufficient resources despite their having a adequate volume of cases. In addition, there are the need for a histopathologist's help with the identification, sampling and characterization of the neoplastic tissue, and to the lack of close follow-up of large series of patients [1, 2].

An analysis of the international literature showed that in many molecular, genetic and proteomic studies the method

used to collect the neoplastic tissue was not completely described or validated [2]. The single protocol currently used by some of the centres participating in the Cooperative Prostate Cancer Tissue Resource is a modification of the technique by Bova *et al.* [3]. This method is complex and excludes, the participation of non-academic centres because it is labour-intensive, requiring notable human resources, infrastructure and consumable products.

Every new method proposed as innovative in the field of tissue storage must present a standardisation of the processes of collection, processing, and storage of the material, given the significant impact that each of these phases can have on the quality of results in both the short-term and the long-term. A simple procedure that does not require a large number of specifically delegated staff or consumable products could contain costs but also improve the quality of the material given the reduction in individual variability and errors inherent in every phase of the process. Cost containment would offer more centres with limited resources the possibility of creating their own banks, with the benefit of increasing the number of sample collection centres in the country and, thereby, reducing the loss of patients. [4] Furthermore, by decreasing the costs of the first phase of the collection, more resources and products would be left for the research phase.

For these reasons we are setting up at our Centre banks of biological samples, tissues and data from patients with prostate cancer. The aim of our project, besides validation of the method of collection and storage of biological samples (blood, blood components and urine), was to validate a biopsy technique for creating a bank of frozen tissues.

Materials and Methods

Selection of Patients

First, we obtained the formal approval of the internal protocol by the ethics committee of the Milano 2 Local Health Authority.

Between September 2008 and November 2008, a preliminary analysis was carried out of the storage processes, focusing on human, material and financial resources, the reproducibility of the method of taking samples, the improvement of the quality of the collection processes, analysis of the quality and quantity of material stored, the method of storage, the definition of the work-flow, the improvement of networking between various groups involved and the definition of roles and responsibilities.

Between November 2008 and March 2010, we enrolled patients who were referred to our Unit with a biopsy-confirmed diagnosis of prostate cancer in order to undergo radical prostatectomy. They agreed to take part in the study after a clear and full clinical interview. They signed an informed consent to the management of their personal data, to the storage of their biological material and to the use of this biological material for research purposes. Patients infected with human immunodeficiency virus, hepatitis B virus or hepatitis C virus were excluded. Patients who had undergone diagnostic biopsy of the prostatic neoplasm at another Institution were also excluded.

Overall, 62 patients were enrolled. In 7 patients the preoperative biopsy showed microfoci of neoplasia and for this reason they were excluded from the sampling protocol; the study was, therefore, conducted on the remaining 55 patients. The mean age of these patients was 62.8 years (range, 52–74 years). Their mean preoperative PSA (Prostate Specific Antigen) was 8.71 ng/ml (range, 3.2–40 ng/ml). The mean weight of the surgically excised prostate was 53.4 g (range, 25–140 g). According to the 2002 TNM classification, 3 patients had pT2a, 4 pT2b, 35 pT2c, 10 pT3a, 2 pT3b, and 1 pT4 [5]. The definitive histological studies revealed malignancy in one lobe in 31 patients and in two lobes in the other 24 patients. The width of the tumour (maximum diameter) ranged between 1 mm and 35 mm.

Databases and Biobank

Each patient's data were recorded in two databases while their biological samples were stored in a biobank:

- Personal information database: this database contains the personal information on each patients and the connection between these data and the printed list of identification codes (alphanumerical) enabling referral to the rest of the data stored in the other database and samples conserved in the biobank.
- Clinical database: this database contains clinical information such as a detailed past and current clinical history, results of the digital rectal examination, preoperative PSA, the prostate biopsy report, any hormone therapy, the results of any investigations carried out to exclude metastases, the histological characteristics of the neoplasm and clinical follow-up investigations. This database can also store video or photographic documentation.
- Biobank: this contains biological samples from each patient, stored for research purposes, and is divided into the following sections:
  - (i) Urine: a 100 ml of midstream urine sample collected during the first passage of urine on the morning of the operation. These samples are kept in a refrigerator until being centrifuged within 30 min of collection at 3,000 revolutions for 10 min. The resultant supernatant is frozen at −80 ° C and stored in 50 cc cyrotubes. [6]
  - (ii) Plasma*:* the sample of plasma is taken on the morning of the operation after an overnight fast. The

samples, collected into EDTA-containing test-tubes, are stored in a refrigerator until being centrifuged at 2000 revolutions for 10 min. The plasma, buffy-coat and the precipitate are removed and stored in 2 cc cryotubes at −80°C. From each EDTA-containing test-tube, three biological samples are obtained: 2 cc of plasma, 2 cc of precipitate and a few drops of buffy coat. From a proteomics perspective, the difference between serum and plasma can be considerable. The activated proteases during the process of activation of the coagulation cascade will in turn have proteolytic effects on other proteins. Hulmes *et al.* showed that addition of a protease inhibitor cocktail directly to plasma collection tubes prior to phlebotomy, centrifugation within 1 h of blood drawn, snap-freezing aliquots immediately and storing frozen aliquots in a −70°C freezer can improve sample qualities for proteomics analysis [7]. EDTA seems to be the anticoagulant of choice for proteomics analysis of body fluids when the primary aim is to catalog and quantitate proteins [8].

(iii) Fresh material from the surgical specimen: we here propose a new method for the collection of this material. The tissue is taken immediately from the excised surgical specimen using the technique described below.
The equipment used for the sampling are: a prostatic biopsy pistol, a vial of India ink, a syringe and a 1 ml Eppendorf tube containing 50 μl of RNA-later (RNAlater® Tissue Collection).
Three targeted biopsies are taken from the area indicated by the previous biopsy mapping (carried out according to the description by Gore) as the site of the neoplasm [9]. Under manual palpation of the excised surgical specimen to identify the neoplastic nodule, the surgeon takes the samples using an 18 Gauge biopsy needle.
The site of the sampling is labelled by injection of India ink in order to allow the histopathologist to determine whether the area involved by the malignant disease was sampled. The correctness of the sampling is determined by taking a biopsy from the same site, which is processed in formalin. In the definitive histological examination, the correctness of the sampling is noted (both by visualisation of the stained area, and by the reading of the biopsy).
After immersion in RNA-later for a few hours, the biopsy chips are stored at −80°C. The same procedure is also used to obtain chips of presumably healthy tissue from the contralateral lobe or from areas not reported as neoplastic; these are processed with the same method. The remainder of the surgical specimen is routinely processed by fixation in formalin for the definitive histological examination.
The validation of this method was obtained from the histopathological confirmation of the correctness of the sampling, by comparing the percentage of samples positive for malignancy with those negative and then comparing the value obtained with that reported in the literature using the method of Bova *et al.* [3].

(iv) Formalin-fixed material: this is constituted by the patient's previous prostatic biopsies and the surgical specimen appropriately fixed in formalin and treated in order to allow histopathological staging of the disease.

Custody of the Samples

Each sample is labelled with the letter P (for prostate) and three digits forming a number from 001 to 999 indicating the numerical progression. The information enabling the relationship between a sample and a patient's identity is kept on computer and in printed form, protected by an access code in order to protect to the non eligible personal the use of these personal data.

Case Report Form

In order to relate data from the databases and samples in the biobank quickly, and create a summary form for each patient, a case report form is compiled (Fig. 1).

Results

One doctor (P.S.), specifically trained in the 3 months before the start of the project, was responsible for the collection, treatment and storage of the samples. In 43 cases, biopsies collected from the tumour site were correct, with a mean of 48% of the core involved by tumour (range, 10–90%). A negative biopsy was observed in 5 cases of mismatch between the previous biopsy report and the definitive report. In seven cases it was not possible to obtain part of the neoplastic lesion from within the chips. The overall tumour detection rate was 78.2% (43/55). Table 1 reports the different detection rates according to the histopathological stage of the disease, and the presence or absence of a palpable nodule to guide the biopsy.

In the two cases in which, in the presence of a palpable nodule, the biopsy did not include neoplastic tissue, histological studies showed fibrosis.

All the samples of blood components and urine were treated and stored within 30 min of collection. The process of tissue collection was simple, easy to carry out, fast and

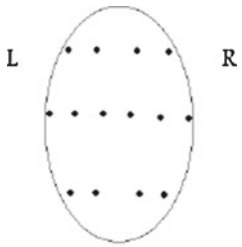
CASE REPORT FORM

- ***General Data***

Patient's code _____
Date of birth ___/___/___
Date of operation ___/___/___

- **Preoperative data**

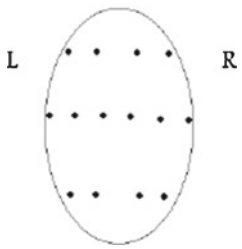
Preoperative PSA _____ ng/dl
Preoperative prostate biopsy



Preoperative Gleason _____
Preoperative staging _____
Preoperative treatment
□ none
□ 5 – HT
□ LHRH analogues

- **Postoperative data**

Postoperative Gleason _____
Postoperative staging _____
Biopsy mapping (indicate site, number of biopsies and location of tumour mass)



Correct sampling
□ Yes
□ No
Quality of sampling
□ Good
□ Poor
Additional samples
□ Urine _____ ml
□ Blood _____

Signature _____
date ___/___/___

Fig. 1 Case report form

Table 1 Results of the sampling method according to the histopathological characteristics of the disease

	Stage	% identification of total	% identification excluding cases of mismatch
Biopsies positive for malignancy divided by pT	pT2a	33%	33%
	pT2b	50%	50%
	pT2c	80%	90%
	pT3a	80%	90%
	pT3b	100%	100%
	pT4	100%	100%
Biopsies positive on the basis of the presence of a palpable nodule	YES	94% (34/36)	
	NO	47% (9/19)	
Overall detection rate	78.2%		

cheap. On average, the tissue biopsy took 3 min and the biopsy was immediately immersed in RNA-later.

In no cases there were problems with histopathological staging of the disease.

In total, ten specimens were sacrificed to determine whether freezing could negatively affect the interpretation of the histological studies of the biopsy chips. The analysis after formalin fixation did not reveal any important microscopic changes related to the freezing or difficulties in processing the sample.

Discussion

The natural history of early stage prostate cancer suggests that many patients with this condition will not die from their tumour. Indeed, the rate of overdiagnosis of prostatic cancer following the introduction of screening has been estimated to be between 27% and 56% [10, 11]. With a mean follow-up of 9 years, the *European Randomized Study of Screening for Prostate Cancer* (ERSPC) reported that PSA screening reduced the mortality rate for this neoplasm by 20%, but also noted that 1,410 men would have had to be screened and another 48 treated for this cancer in order to prevent one death [12].

Improving knowledge concerning this disease, in particular by increasing the availability of biological, histopathological and clinical data available for people involved in research, could be of precious help in optimising treatment outcomes. Tumour banking offers the promise of treating specific serious illnesses through understanding the cellular basis of disease and thus developing targeted therapies.

In recent years, numerous tissue banks have been established and operate using standard procedures in order to reduce the variability related to the collection of samples for research purposes. In fact, variability in the different methods of collecting, treating and storing samples can influence the reproducibility of research procedures and analyses. The policy of different committees reviewing biological specimens led to the conclusion that efforts to harmonise the processing of samples is an area of significant importance for improving the inventory of biobanks [1]. There are several variables involved in the process of collecting samples from surgical specimens. These can be divided into clinical and non-clinical variables. The aim of our work was to reduce the non-clinical variables acting between amputation of the tissue and sampling of biological fluids and their storage. There is a general consensus within biobanks that this period should be reduced as much as possible, with 30 min being considered the gold standard.

Our internal protocol for the collection of biological material and the innovative method for collecting the fresh tissue both reduce the internal steps and staff involved between the surgical amputation of the specimen and the storage, thereby reducing all the variables related to the processing of the samples that could lead to heterogeneity in the material and changes in its quality. If the staff directly involved in the collection of the samples are correctly trained and educated on the methods of treating and storing the sample, with simple and standardised procedures, there will be greater efficiency in terms of time, human resources and financial resources.

In 1999, the National Cancer Institute (NCI) recognised the need for a multicentre effort regarding biobanking of prostate cancer biological material [13]. In April 2000, four academic institutions were funded to form a national prostate cancer tissue resource, the Cooperative Prostate Cancer Tissue Resource (CPCTR). The technique of sectioning the radical prostatectomy specimen used at most of the sites involved in the CPCTR is a modification of the technique described by Bova *et al.* in 1993 [3]. Briefly, this technique consists in marking the surface of the gland with India ink, before the capsule is stripped from the gland and alternate whole 0.5-cm-thick slices of the specimen are routinely procured, snap-frozen and stored at −150°C in vapour phase liquid nitrogen freezers. Each slice is typically quartered (approximately 2×2 cm) before freezing. To date, quality assurance examination has demonstrated that tumour can be

identified and isolated from frozen tissues in approximately 70% of the cases. This method is time-consuming and can only be done in structures with considerable financial and human resources. If the initial phases of processing the surgical specimen are not carried out correctly, there can be repercussions on the histopathological staging. Furthermore, every frozen tissue block must be newly reviewed in order to determine whether it contains tumour tissue.

The biopsy method that we propose for the purpose of collecting tissue samples is fast (which means the degradation of RNA and proteins is minimised), does not require additional staff or particular products for the immediate treatment of the sample, does not compromise the correct histopathological staging of the disease, does not require further histological confirmation on every chip collected, does not require large amounts of space for storage and provides a high percentage of stored tumour tissue available in 78% of the cases. No less importantly, no samples need to be sacrificed, even the incorrect ones, since they can be the source of tissue from the peri-tumoral zone.

The RNA-later eliminates the need for immediate freezing of the sample, enabling storage and indefinite protection of the RNA and the possibility of incorporate the chips into tissue microarrays, obtaining up to 150 consecutive sections from a single chip in order to increase the potential of studies of biomarkers and immunohistochemistry [14]. Tissue microarrays are ideal for efficient screening of prospective biomarkers by a variety of different mechanisms including immunohistochemistry, fluorescence in-situ hybridisation of nucleic acids and RNA in-situ hybridization. These techniques help in tumour profiling, rapid screening of gene amplifications in cancer, verifying *in-vivo* differential expression of genes identified by cDNA arrays, and identifying prognostic and diagnostic markers.

Our proposed new protocol is compatible with all the six critical functions that should be served by a prostate biobank including accrual of normal and neoplastic prostate samples, banking of blood and blood components and other biological specimens, triage of fresh tissue for a variety of projects to provide fresh normal and neoplastic tissues, data collection facilities, morphology support, and human subjects and Institutional Review Board issues [1].

Conclusions

The protocol was developed in order to provide high quality, low cost material for research on prostate cancer. Besides emphasizing the importance of the validation of a protocol for collecting, treating and storing biological material, it is also important to underline the need for the protocol to be simple, reproducible and cheap. It provides a high percentage of stored tumour material available in 78% of the cases. The characteristics of the protocol enable its use also in non-academic organizations, which do not have major research funds, but which can actively contribute to the creation of a network of centres able to collect good quality material for basic research on prostate cancer. The same approach could be useful in other oncological disease of different organs including thyroid, adrenal gland, kidney, lung and for store cancer cell obtained from metastatic lymph-nodes. The possibility of obtaining fresh cancer material, without alteration of the pathological staging of the disease, appears attractive for future applications in different anatomical and pathological area for molecular and genetic study.

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