High quality RNA isolation from tumours with low cellularity and high extracellular matrix component for cDNA microarrays: application to chondrosarcoma

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Abstract
Aims—High quality RNA isolation from cartilaginous tissue is considered difficult because of relatively low cellularity and the abundance of extracellular matrix rich in glycosaminoglycans and collagens. Given the growing interest and technical possibilities to study RNA expression at a high throughput level, research on tissue with these characteristics is hampered by the lack of an efficient method for obtaining sufficient amounts of high quality RNA.
Methods—This paper presents a robust protocol combining two RNA isolation procedures, based on a combination of Trizol and RNA specific columns, which has been developed to obtain high molecular weight RNA from fresh frozen and stored tissue of normal cartilage and cartilaginous tumours. Using this method, RNA was isolated from normal cartilage, peripheral chondrosarcoma, and central chondrosarcoma.
Results—The yields ranged from 0.1 to 0.5 μg RNA/mg tissue. RNA isolated with this method was stable and of high molecular weight. RNA samples from normal cartilage and from two chondrosarcomas isolated using this method were applied successfully in cDNA microarray experiments. The number of genes that give interpretable results was in the range of what would be expected from microarray results obtained on chondrosarcoma cell line RNA. Signal to noise ratios were good and differential expression between tumour and normal cartilage was detectable for a large number of genes.
Conclusion—With this newly developed isolation method, high quality RNA can be obtained from low cellular tissue with a high extracellular matrix component. These procedures can also be applied to other tumour material.
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Keywords: RNA extraction; cartilage; bone neoplasm; chondrosarcoma; cDNA microarray; expression profile

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Molecular genetic studies on cartilaginous tumours described in the literature mostly involve DNA (for example, microsatellite analysis, mutation analysis, and comparative genomic hybridisation) or proteins (immunohistochemistry). Studies on RNA are sparse. RNA isolation yielding appropriate amounts of high molecular weight RNA from cartilaginous tissue is difficult because of low cellularity and the abundance of extracellular matrix material, which is rich in glycosaminoglycans and collagens. In addition, part of the material may be calcified. Classic RNA isolation methods give a low yield and unstable products, which degrade quickly. Previously, we have detected gene expression in chondrosarcoma using conventional RNA isolation procedures, but using reverse transcription polymerase chain reaction, which is extremely sensitive and requires only small quantities and short RNA fragments to give good results (our unpublished data).

With upcoming novel techniques, such as cDNA microarray, researchers working on cartilaginous tissues are being confronted with problems regarding the isolation of RNA from these tissues. The cDNA microarray technique uses the wealth of data on known and novel genes that is becoming available from the Human Genome Project. Detailed expression profiles can be constructed within a single experiment and provide data on the RNA expression of thousands of genes.[1] The applications of this technique have recently been reviewed.[2 3] For this procedure large quantities of high quality RNA are necessary.

Apart from some extremely rare variants, such as clear cell and mesenchymal chondrosarcoma, two subtypes of conventional chondrosarcoma can be distinguished: central (arising centrally in bone) and peripheral chondrosarcoma (arising within the cartilaginous cap of a sporadic or hereditary osteochondroma (exostosis)). Although these tumour types are similar at the cytonuclear level, we have shown that a diverging genetic mechanism is operative in the origin of central and peripheral chondrosarcoma.[4] Peripheral chondrosarcomas were characterised by genetic instability, as demonstrated by a broad range in DNA ploidy, including near haploidy,[4 5] and a high percentage of loss of heterozygosity involving many different chromosomes. The EXT genes, involved in hereditary multiple exostoses syndrome (HME, EXT), were shown to play a role in peripheral tumour formation.[4 6 7] Osteochondroma of patients with HME showed next to germ line mutations loss of the remaining wild-type allele in the cartilaginous cells, implying a tumour suppressor function for

EXT1 in these lesions.[6] In contrast, central chondrosarcomas were mostly peridiploid and had limited loss of heterozygosity and no alterations in EXT1 or EXT2.[4]

To elucidate further the genes or pathways involved in the development of these two types of chondrosarcomas it would be useful to construct gene expression profiles of these tumours using cDNA microarrays. To render cDNA microarrays and other large scale expression studies accessible for normal cartilage and chondrosarcomas we have developed a method that combines several RNA purification steps and results in high quality and high molecular weight RNA. Here, we show that this RNA can be used in cDNA microarray experiments. In this pilot study, the expression patterns of both primary tumour material and chondrosarcoma cell lines are compared.

Materials and methods

TISSUE SAMPLES

Tumour material obtained from resected specimens of a peripheral and a central chondrosarcoma was snap frozen in CO_2, cooled in 2-methyl-butane, and stored at −70°C until use.

Cartilaginous tissue from the cartilago costalis of a stillborn child obtained after necropsy was used as normal tissue. This material was obtained in accordance with the guidelines of the Dutch Federation of Scientific Societies (FEDERA) and stored anonymously. From this normal cartilage sample, all surrounding tissues including periosteum were removed. Fresh frozen tissue blocks were sectioned on a cryostat. One section of 5 μm was mounted on a glass slide and stained with haematoxylin and eosin for histological examination, and to confirm the absence of contaminating non-cartilaginous normal tissue.

Two chondrosarcoma cell lines were used, SW1353, obtained from the ATCC, and OUMS-27, kindly provided by Dr Namba (Okayama Medical School, Shikata, Japan).[8] There is no record of the subtype (central or peripheral) of the primary tumour from which these cell lines have been derived.

RNA ISOLATION

RNA was isolated using a combination of two RNA isolation procedures. From frozen tissue, 20 μm sections were made and collected in a frozen tube. Approximately 250 mg frozen material was dissolved in 5 ml Trizol (GibcoBRL/Life Technologies, Breda, the Netherlands) and homogenised with an ultraturrax for one minute. When less than 2 μg RNA was expected, 10–20 μg glycogen was added to the suspension. After adding 1 ml chloroform and mixing for one minute, the suspension was centrifuged at 15 000 ×*g* for 10 minutes. A second phenol/chloroform extraction was performed, followed by an isopropanol precipitation. The air dried pellet was dissolved in 100 μl double distilled water and further purified with a RNeasy mini column (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions.
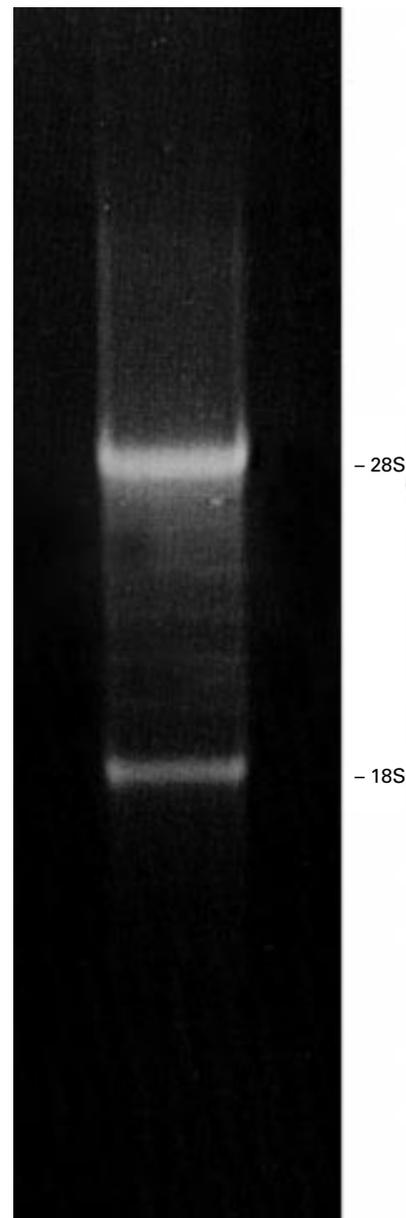

Figure 1 Example of RNA isolated from chondrosarcoma run on a formaldehyde/agarose gel and stained with ethidium bromide. 28S and 18S ribosomal RNA bands are indicated.

The isolated RNA (1 μg/lane) was electrophoresed for three hours at 50 V on a 1% agarose/formalin gel, and stained with ethidium bromide to assess the quality of the RNA.

RNA from the two chondrosarcoma cell lines was isolated using the standard Trizol procedure and used for comparison with the primary tissue samples.

MICROARRAYS

We applied the commercially available microarray system: Micromax™ human cDNA system 1.1–TSA (NEN/Perkin Elmer, Zaventem, Belgium). This system consists of slides containing control and housekeeping genes, cDNA arrays containing 2400 known genes, and reagents for labelling of reverse transcribed RNA and for hybridisation of the arrays. Labelling and hybridisation were performed

Table 1   Results of four cDNA microarrays with chondrosarcomas (CS)

	1	2	3	4
Cy5 sample	OUMS cell line	Peripheral CS	Normal cartilage	Normal cartilage
Cy3 sample	SW1353 cell line	Central CS	Central CS	Peripheral CS
Mean signal/no flags: Cy5*	3078/4026	1299/1947	907/1192	949/1377
Mean background/no flags Cy5*	499/486	575/560	627/5726	662/686
Mean signal/no flags Cy3*	3095/4077	1155/1842	440/613	489/817
Mean background/no flags Cy3*	512/527	402/405	344/340	267/274
No. of flags†	662	1150	1448	1452
Range of ratios‡	49–0.067	46–0.053	22–0.061	27–0.021
Cy5/Cy3 ratios >3	236	57	31	23
Cy5/Cy3 ratios >2	421	130	93	109
Cy3/Cy5 ratios >3	225	59	31	76
Cy3/Cy5 ratios >2	438	155	65	125

*Non-normalised signal obtained by analysis with GenePix 3.0 package; "no flags" refers to the mean signal of only the reliable spots on the array.
†Number of spots that were not detectable because of weak signal or bad signal to noise ratio.
‡Ratio of the Cy5 versus the Cy3 ratio, normalised according to the GenePix 3.0 program.
Cy3, fluorescence at 532 nm; Cy5, fluorescence at 635 nm.

according to the manufacturer's instructions. In brief, 8 µg of total RNA was converted to cDNA and labelled with biotin or dinitrophenyl (DNP). A 4 µg sample was used for test hybridisation on the practice slides. The remaining 4 µg samples of labelled cDNAs were pooled and simultaneously hybridised overnight on a 2400 gene microarray. Biotin and DNP cDNAs were detected with a series of conjugate reporter molecules using the tyramide signal amplification (TSA) system.[9]

An Affymetrix/GMS 418 scanner (MWG-Biotech AG, Ebersberg, Germany) was used to detect the fluorescence at 532 nm (Cy3) and 635 (Cy5). Scanning data were analysed with the GenePix Pro 3.0 analysis program (Axon Instruments, Foster City, California, USA).

Results

RNA ISOLATION

Sections of 20 µm thickness were used for RNA isolation. This ensured proper grinding of the tissue without applying too much force or producing a rise in temperature. The subsequent double purification, first using an RNA specific extraction method (Trizol), followed by an RNA specific column (Qiagen), adequately removed the excess of extracellular matrix from the sample. Application of only the Trizol method resulted in RNA that degrades quickly, with a 260/280 nm ratio of less than 1.5, and which is contaminated with genomic DNA. When only the column is used, yields were extremely low, most probably because of the limited capacity of the column and the abundant amount of extracellular matrix material passing through. The whole purification procedure as proposed in our study could be completed within four hours, thereby reducing the handling period, and thus degradation time, to a minimum.

The amount of total RNA that could be extracted from primary cartilage tissue ranges from 0.1 to 0.5 µg/mg tissue, with a 260/280 nm ratio between 1.9 and 2.1. Figure 1 shows an agarose gel with a typical RNA isolation from a central chondrosarcoma.

cDNA MICROARRAY HYBRIDISATION WITH CARTILAGE RNA

RNA from normal cartilage and from two chondrosarcomas was labelled and hybridised with cDNA microarrays using the Micromax system. Four different hybridisations were performed (summarised in table 1). Figure 2 shows the image of the microarray hybridised with central chondrosarcoma and normal cartilage. The first array in table 1 represents results obtained with two different chondrosarcoma cell line RNA samples. The other three arrays were all hybridised with RNA from primary, fresh frozen tissue. This pilot experiment showed that we could generate cDNA microarray data using RNA isolated from fresh frozen primary cartilage and chondrosarcoma tissue.

As a general rule, signals should be at least 1.5 times higher than background values to obtain reliable measurements. Table 1 shows that this is indeed the case, especially for the data that are not "flagged" by the GenePix analysis program. Flagging is a function of the program and is performed automatically for those spots that have too low an intensity for both dyes to be detected. In the three arrays hybridised with primary tissue, 46%, 60%, and 60% of the signals were "flagged". This may be because many genes on this standard array are not expressed in cartilaginous tissue. Most of the cDNAs that were flagged in the chondrosarcoma cell line array (n = 662) were also flagged in all three other arrays (n = 380) or in two arrays (n = 145). When flagging in the
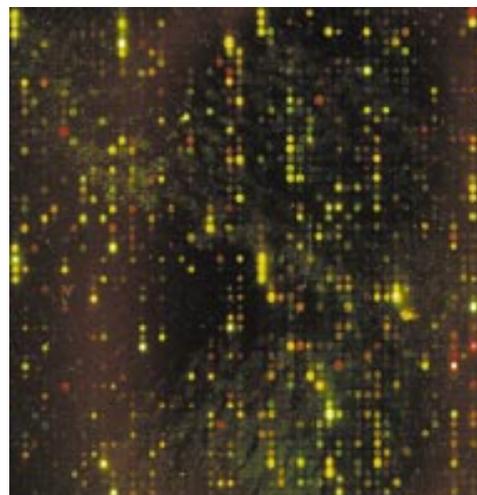

Figure 2   Example of cDNA microarray hybridised with primary tumour RNA of a central chondrosarcoma and normal cartilage.

three arrays hybridised with RNA from primary tissue were compared, again most flags were assigned in all three experiments (n = 878). Furthermore, the array hybridised with RNA from chondrosarcoma cell lines showed substantially fewer flags than the primary tissue. This probably reflects the high degree of gene expression in exponentially growing cultured cells.

Table 1 shows that a considerable number of genes were differentially expressed in tumour tissue compared with normal tissue, and when central and peripheral chondrosarcomas were compared; thus, this technique could be used to identify genes that are upregulated or downregulated in the two tumour types.

Discussion

With the new techniques that have emerged for large scale expression studies (such as cDNA microarraying) there is now a need for high quality RNA from primary tissue. We have developed a reliable method for the isolation of high quality RNA from fresh frozen cartilaginous tissue obtained from clinical samples—postmortem tissue or surgically retrieved tissue. Previous reports on RNA isolation from cartilage are mostly on experimentally obtained material from laboratory animals or from cultured cells. Logistic problems cause delay in sampling and do not allow direct RNA isolation from clinical samples, which are stored at −70°C for several weeks, months, or even years. The method described here is suitable to obtain high quality RNA from this material. This was shown by successful application of the novel cDNA microarray technique. The labelling method used entails an amplification of the final fluorescence signal using the TSA system,[9] which requires only 4 μg total RNA, instead of the usual 100 μg.[10] We show that the signal to noise ratio is good enough for detection of gene expression differences and that the differences found are substantial.

The initial goal of the microarray hybridisation described here was to show that our RNA isolation method is suitable for highly demanding techniques, such as cDNA microarraying. To draw conclusions on the results of this experiment, repeat experiments and additional patient samples to confirm these results are necessary. Because these standard commercial arrays lack many genes that are relevant for cartilage development and chondrosarcoma, we are currently developing custom arrays that contain such genes—for example, the genes of the EXT family and their supposed partners in signalling, especially Hedgehog and genes involved in heparan sulphate regulation.

However, there are some noticeable differences in expression seen in our present experiment. The interpretation of these results needs to be corroborated by repeating the experiments and the use of robust statistical methods. Nonetheless, some of the results obtained so far are very interesting in view of recent knowledge obtained on the molecular pathways operative in the genesis of cartilaginous malignancies.[11] Different clones with related gene function on the array gave similar results in separate hybridisation experiments. A pronounced overexpression of α actin and its crosslinking partner α actinin was found in peripheral chondrosarcomas when compared with central chondrosarcomas (five times and 15 times greater expression, respectively) or with normal cartilage (36 times and 13 times, respectively). Recently, overexpression of the actin protein was found in chondrocytes and osteochondromas with mutations of EXT1 or EXT2, which are only detected in patients with hereditary multiple osteochondromas that may become malignant peripheral chondrosarcoma.[12 13] Even more pronounced is the overexpression of myosin, the motor protein of actin: 25 times when comparing peripheral chondrosarcoma with central or 52 times when compared with normal cartilage.

Other prominent differences are overexpression of immunoglobulin related genes in peripheral chondrosarcoma: light chain expression 46 times and eight times higher than in central chondrosarcoma and normal cartilage, and heavy chain expression was 27 times and 47 times higher, respectively. Central chondrosarcoma shows a pronounced overexpression of almost all ribosomal proteins on the array when compared with peripheral chondrosarcoma; however, when compared with the normal cartilage, peripheral chondrosarcoma shows consistently higher expression of these genes.

Overexpressed genes may be potential oncogenes in these tumours. Previously, we have shown pronounced differences in the tumorigenesis of central versus peripheral chondrosarcoma at the molecular genetic level.[4] Our current experiments also detected differences in expression pattern between these two types of cartilaginous malignancies.

In conclusion, by developing an RNA isolation protocol for cartilaginous tissue we have rendered these difficult tumour tissues available for large scale expression studies. This method may also be suitable for RNA isolation from other tissue types that are difficult to homogenise, have poor cellularity, or have a high extracellular matrix content.

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