CLINICAL RESEARCH

DNA EXTRACTION USING CHELEX RESIN FOR THE ONCOGENIC AMPLIFICATION ANALYSIS IN HEAD AND NECK TUMOURS

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ABSTRACT

DNA extraction from tissues can be the most laborious and complex step in amplifying DNA by PCR when phenol-choroform procedure is used. We compare this lengthy, slow and expensive extraction method with other two based in the use of Chelex-100 resin. This chelating resin has been applied for extracting DNA from different tissues to use with the PCR. These procedures are simple, rapid and do not require multiple steps. In this study we compared DNA extraction from 30 head and neck squamous cell carcinomas (HNSCC) using organic solvent precipitation, Chelex 100 resin with and without proteinase K pretreatment. The results show that proteinase K-Chelex 100 procedure is as efficient as the phenol-chloroform one.

KEY WORDS: Head and neck cancer. Fibroblastic growth factors. DNA extraction. Oncogene amplification. Polymerase Caín reaction. Chelex 100-resin.

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INTRODUCTION

What we currently know about molecular alterations in carcinogenesis has allowed us to obtain information about the possible evolution and prognosis of tumours. What is best known about the genetic characteristics of tumours relates directly to the advances of techniques in molecular biology. The application of the technique of polymerase chain reaction (PCR) allows for the detection of specific DNA sequences without the need for fragments of a high molecular weight; it achieves detection with high specificity and proves to be an optimum method for the simultaneous processing of a large number of samples. These samples of DNA may derive from small quantities of tumour (surgical pieces, biopsies, paraffin samples, tissue taken by means of punction aspiration). Complementarily, it is desirable to use a simple method of DNA extraction that avoids contamination of samples; this is a possible drawback of the method of precipitation with phenol/chloroform. There are DNA extraction techniques, such as incubation with SDS[1,2] or sonication[3], which are fast and easily applied but require large quantities of tissue. In this paper we used Chelex-100 resin, a cation chelating resin used for DNA extraction, both with and without previous digestion by proteinase K. These procedures have been compared with results obtained from purification with organic solvents and precipitation with ethanol. Even though there are previously existing studies about the usefulness of DNA extraction with Chelex-100 resin[4-9], studies of its clinical application are scarce[10]. In this paper we have analysed the rate of amplification of oncogenes *INT2* and *HST1,* which belong to the family of fibroblast growth factors. These are linked to the process of tumoral angiogenesis, present in the development of ganglionic metastasis in epidermoid carcinomas of the head and neck[11].

MATERIAL AND METHODS

Tumour samples and DNA extraction

Tumour samples from 30 epidermoid head and neck carcinomas were obtained during the surgical resection of the tumour. A portion of tumoral tissue without necrotic areas was obtained from the surgical piece and was immediately frozen and stored in liquid nitrogen at -70 ˚C until DNA extraction. The tumour samples were cut and processed in accordance with the following DNA extraction methods (A-C):

Method A

This method consists of DNA extraction by means of the classic method of precipitation with phenol/chloroform[12], observing the following steps. Firstly, the frozen sample, with an average size of 10 x 10 mm, is carefully cut into small pieces. It is then homogenized by being shaken in 5 ml of extraction buffer (Tris-HCl 0.02 M pH 8.0, EDTA 0.025 M, NaCl 0.1 M and SDS 0.2%). 10 µl of RNAase (10 mg/ml; Boehringer Mannheim) is added to the mixture which is then incubated at 37˚C for an hour while being continually shaken. The next step is to add 20 µl of proteinase K (25 mg/ml; Boehringer Mannheim) and incubate the mixture while shaking it constantly for 5 hours at 37˚C. This step is repeated twice. The solution is then allowed to cool to room temperature and an equivalent volume of phenol equilibrated with Tris-HCl, 0.5 M pH 8.0 is added to it. The solution is then centrifuged for 15 minutes at 2500 rpm, thus forming a lower organic phase and an upper liquid phase. The liquid phase is drained off into a tube where an equivalent volume of isoamyl alcohol/phenol/chloroform is added to it. This mixture is centrifuged for 15 minutes at 2500 rpm. The supernatant obtained is diluted in two volumes of isopropanol at –20˚ C. At this stage the DNA is precipitated and extracted. The precipitated DNA is washed in ethanol at 70%. Finally, the DNA is dried and dissolved in 500 µl of Tris-EDTA 10:1 and the solution is stored at -20˚ C.

Method B

A tissue sample (of approximately 1x1x1 mm) is introduced into an Eppendorf tube and mixed into 500 µl of 10% Chelex-100 resin solution. This solution has been previously prepared by dissolving and stirring the designated quantity of resin into sterilized deionized water at 60˚C. 15 µl of proteinase K (Boehringer Mannheim) at 10 mg/ml is added to the solution which is then incubated at 55˚ C for an hour while being shaken. The mixture is then incubated further at a temperature of 100˚C for 15 minutes to increase the extent of protein denaturation. The tubes containing the extracted

DNA and the Chelex resin, together with the degraded proteins, are stored at 4˚ C or at -20˚ C. Before it is used the mixture is shaken in a vortex mixer and centrifuged at 10,000 rpm for ten seconds to deposit the mass formed by the resin and the proteins. The tube is subsequently centrifuged again for 10 minutes to separate the surface layer in which the DNA can be found and the lower layer which contains the Chelex-100 resin, the denatured proteins and other elements.

2 µl of supernatant are used for each 10 µl of final volume of the PCR reaction mixture.

Method C

This method uses the same protocol but proteinase K is not added into the mixture of tissue and Chelex-100 resin.

Oligonucleotide Primers and PCR analysis. The mixture for the PCR contains 0.2-0.5 µg of DNA, 10 mM Tris-HCI pH 8.3, 1.5 mM MgCI2, 50 mM KCI, 0.2 mM of each dNTP, 1 µM of each primer, 1 µM and 1 U of Taq polymerase (Boehringer Mannheim, Mannheim, Germany) in a total volume of 50 µl. 50 µl of mineral oil is then deposited over the mixture. The PCR process involves 30 cycles, each comprising 1 minute at each of the following temperatures: 94˚C, 56˚C and 72˚C. This is followed by a final 7 minute incubation at 72˚C. Two different types of primer, one for the genes to be studied (*INT2* and *HST1)* and another for the gene that was used as a study control (tyrosine hydroxylase) are introduced into the mixture of the reaction of the PCR differential[13]. In our study we used the tyrosine hydroxylase gene because it is localized in the same chromosome as the oncogenes being analysed. The primers were designed from genome sequences from GenBank. The primers for the oncogene *INT2* (GenBank accession number NM005247) were 5'- TGG AGG TGG GCA TTG TGG- 3' and 5'- ACC GCT ACT CCG TCA GCG.-3'. The amplified fragment consisted of 128 base pairs. For its part, the primers of oncogene *HST1* (GenBank accession number MN002007) were 5'- TGA GCA TCT TGC GCG TGG - 3' and 5'- GCC ACG AGC CTG CTA GCC -3', amplifying a DNA fragment of 136 base pairs. The primers of the gene TH (GenBank number D00269) were 5'- GCC CCA GCT GCA TCC TAC- 3' and 5'- CTT GGC AGA CAC CTG GGG -3' amplifying a DNA fragment of 188 base pairs. The primers were obtained from the MWG-Biotech laboratory (Mannheim, Germany). Samples of normal tissue (tonsils) obtained from non-smoker patients were used as negative controls. As positive controls we used a mixture of normal tissue DNA with different quantities of amplified sequences of the oncogenes under investigation, which simulated different degrees of amplification.

Electrophoresis and quantification of the results of the PCR. After the PCR, 10 µl of each sample was subjected to electrophoresis on 3% gels made from NuSieve agarose (FMC, Rockland, ME) for 1.5 hours at 65 V in a 40 mM Tris-Acetate, 2 mM EDTA buffer. The gels were stained with ethidium bromide and the images under ultra violet light were captured by a digital camera and stored in a computer. The bands that were shown up by the gels were quantified by means of computerized densitometric analysis systems (Kodak Digital Science 10, Eastman Software, Billerica, MA). In this way we obtained ratios of amplification of the oncogenes *INT2* and *HST1* in comparison with the control gene, TH. The results taken from the densitometer and the quality of the reaction (presence or absence of unexpected bands) were inspected taking as a reference the markers of molecular weights introduced into the gels.

RESULTS

The PCR amplification of fragments of 128 base pairs corresponding to oncogene *INT2,* fragments of 136 base pairs corresponding to oncogene *HST1* and of fragments of 188 base pairs belonging to gene TH, were analysed using the three methods previously described on the 30 tumour samples. In the analysis of the gels, the presence of non-specific bands and the ratios of amplification *INT2/TH* and *HST1/TH* were evaluated. For experimental purposes, genetic amplification is defined as an increase of the ratio target gene/control gene greater than 2, in comparison with the proportions in normal tissue, taken as negative controls. Following the classic method A as a reference, 17 (56.6%) of the 30 samples showed amplification of oncogene *INT2* (ratio *INT2*:TH between 2 and 15.6%). For oncogene *HST1*, 15 of the samples

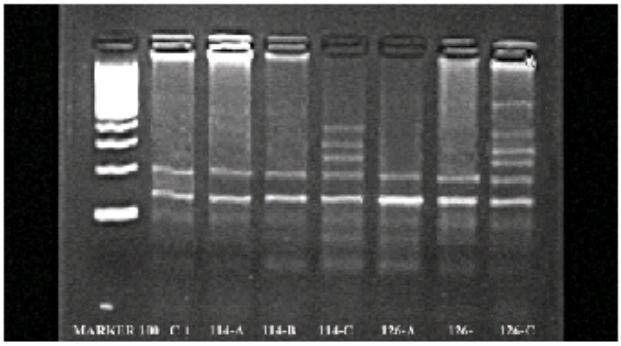

Figure 1. PCR differential of the amplification of oncogene *HST1* for various tumours using the three methods under investigation. The densitometric analysis of the images shows equivalent results, but qualitatively non-specific bands appear in the samples in which method C was used.

showed amplification (50%) (ratio *HST1*:TH between 2.5 and 12.4).

Using method B the results obtained were equivalent to those found with the classic protocol of precipitation with organic solvents. These results were quantitatively and qualitatively objectified. The rate of amplification with oncogene *INT2* was of 3.75 in the samples treated with method A while it was 3.78 in those in which the protocol with Chelex resin and proteinase K (table 1) was used. In the study of oncogene *HST1*, treatment with phenol/chloroform obtained a rate of amplification of 3.37, while for that which used the technique of resin and digestion with proteinase K the rate of amplification was 3.44. From this qualitative point of view no significant differences were found, being no presence of non-specific bands.

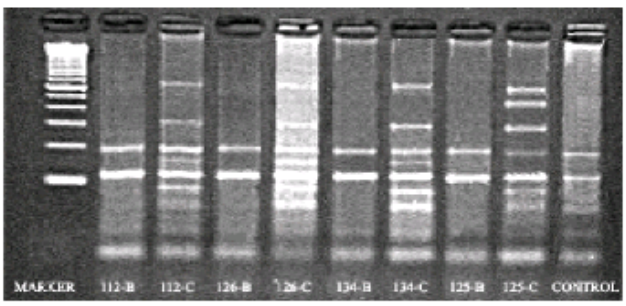

Figure 2. Image of tumour samples studied for the amplification of oncogene *INT2* comparing methods B and C. Quantitatively there is a slight proportion of *INT2/TH* amplification when the sample was not previously treated with proteinase K and there are some non-specific bands in the agarose gel.

The third technique employed, treatment with Chelex-100 without digestion with proteinase K, is simpler and faster than the others but did not produce as satisfactory results as with the other techniques for the two genes studied. We found the presence of non-specific bands and great variation in the rates of amplification in comparison with the results obtained using the other two methods. For oncogene *INT2* the average amplification was 3.44 while for oncogene *HST1* it was 3.23. These results show less efficiency when compared with the previous methods. This variation is more evident in the case of the samples with higher rates of amplification. When Method C was used the quality of the gels obtained was inferior to that of those obtained in either of the other two procedures. This relates to the presence of non-specific bands of varying intensity that made interpretation of the results difficult and ambiguous.

DISCUSSION

Although precipitation with phenol/ chloroform provides DNA fragments of high molecular weight, sufficient for complex investigations of molecular biology, this method involves many slow and costly phases. This process can be difficult to carry out on a large scale in clinical practice[14]. Currently, one of the main applications of this technique is in PCR which can be used on small samples and obtain adequate results with small DNA fragments[5,7,8]. In clinical practice the aim of using the PCR method is to look for molecular characteristics of DNA relating to prognosis, the ultimate objective being to apply the most appropriate treatment for each patient. In these situations it is best if the sample is small in size, taken from a biopsy or even obtained by means of punction aspiration[10]. The purpose of this study is to compare the efficiency of methods of DNA extraction using Chelex-100 resin in comparison with the classic method of precipitation using organic solvents. Chelex resin acts as a chelating resin of metallic polyvalent ions that would act as catalysts in the rupture of DNA at high temperatures thus allowing for inhibition of PCR[10,15]. Previous studies show that the use of Chelex resin without proteinase K provides a quantity of DNA that is sufficient for amplification [7]. However, these studies also indicate some limitations regarding the size of the products that can be amplified. It might be possible to amplify

Table 1: Ratios of amplification of the studied samples with each one of the three methods. The asterisks indicate those samples that showed the presence of non-specific bands. 1 represents the cases that showed amplification and 0 the cases in which it was not shown. In the case of oncogene *INT2* non-specific bands were found using method C in 244 cases while these were present in 21 cases for oncogene *HST1* (70%).

No. of cases	Amplification	AMPLIFICATION *INT-2* Method A	Method B	Method C	Amplification	AMPLIFICATION *HST-1* Method A	Method B	Method C
101	1	15.6	15.8	13.7*	1	12.4	12.56	11.8*
110	0	1.64	1.67	1.58	0	1.52	1.56	1.58
111	1	2	2	2*	0	1.8	1.79	1.77*
112	1	5.3	5.2	4.2*	1	5.4	5.61	5.21*
113	1	3.1	3	2.89*	1	2.6	2.63	2.67*
114	1	4.7	4.81	4.42*	1	3.9	3.98	3.72*
115	1	5.6	5.66	5.11*	1	4.6	4.71	4.66*
116	0	1.76	1.77	1.68	0	1.51	1.5	1.44
117	0	1.85	1.89	1.75*	1	2.24	2.3	2.1*
118	1	4.9	5	4.61*	1	5.1	5.26	4.86*
119	0	1.2	1.2	1	0	1.51	1.54	1.5
120	0	1.45	1.43	1.24*	0	1.62	1.65	1.64*
121	0	1.36	1.35	1.33*	0	1.5	1.53	1.5*
122	1	2.18	2.2	2.21*	0	1.65	1.68	1.61
123	1	7	7.2	6.51*	1	7.8	7.95	6.84*
124	1	3.4	3.43	3.05*	1	2.9	3	2.86*
125	1	7.52	7.57	6.4*	1	6.57	6.61	6.49*
126	1	8	8.21	8.68*	1	6.1	6.31	5.65*
127	0	1.84	1.86	1.8*	0	1.75	1.78	1.72*
128	1	4.4	4.3	3.74*	1	3.6	3.68	3.35*
129	0	1.36	1.37	1.32	0	1.42	1.47	1.44
130	0	1.69	1.74	1.6*	0	1.65	1.7	1.64
131	1	3.15	3.23	2.67*	0	1.69	1.7	1.7*
132	0	1.63	1.67	1.59*	0	1.59	1.6	1.56*
133	0	1.58	1.56	1.54*	0	1.32	1.35	1.29
134	1	9	8.94	8.1*	1	7.8	8	7.12*
135	0	1.26	1.31	1.27	0	1.38	1.4	1.36
136	1	3.7	3.78	3.41*	1	4.1	4.07	3.86*
137	1	2.7	2.8	2.24*	1	2.5	2.64	2.27*
138	0	1.64	1.69	1.62*	0	1.84	1.83	1.78
Average		3.75	3.78	3.44		3.37	3.44	3.23

fragments of up to 650 base pairs in samples treated only with Chelex-100 resin and fragments of up to 1000 base pairs in those in which resin and digestion with proteinase K is used. All the fragments of DNA that were amplified in this paper were small in size (128-188 base pairs) for which reason the application of the methods based on Chelex-100 resin did not present this technical difficulty. Some studies have made manifest a decrease in the amplification of fragments larger than 600 base pairs when samples stored with proteinase K were used, possibly due to a greater rupture of the DNA[16].

The coincidences in the results produced by methods A and B, despite not being described in fresh tissue samples, had been described though in blood cells[17,18], material for forensic study[4,6,19,20], microbiological samples[21-23], solid tissue[24,25], paraffinated tissue samples[26], cervical tumour samples[7] and even human saliva[27].

CONCLUSIONS

We have proven that smaller quantities of tissue, equivalent to tissue samples, are needed when using Chelex-100 resin. This is generally not possible when the samples are processed using method A. Furthermore, the labour intensiveness of the process, and the many steps it involves, make this protocol inappropriate/inadequate for routine and simultaneous application to a large number of cases. Our findings show a strong quantitative and qualitative correlation between methods A and B, which allows for faster and more efficient amplification studies to be carried out with these methods.

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