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USE OF ROUTINELY PROCESSED ARCHIVE TISSUE SAMPLES IN MOLECULAR GENETIC DIAGNOSTICS

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SZTE Egyetemi Könyvtár SZEGED

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LIST OF PUBLICATIONS

Publications:

I) **Sepp R,** Szabó I, Uda H, Sakamoto H. Rapid techniques for DNA extraction from routinely processed archival tissue for use in PCR. *J Clin Pathol* 1994; 47:318-23. **(IF: 1.755)**

II) Szabó I, **Sepp R,** Nakamoto K, Maeda M, Sakamoto, H, Uda H. Human papillomavirus not found in squamous and large cell lung carcinomas by polymerase chain reaction. *Cancer* 1994; 73:2740-4. **(IF: 3.611)**

III) **Sepp R:** Mutációanalízis hypertrophiás cardiomyopathiában. *Cardiologica Hungarica* 1997; 5(Suppl): 15-18.

IV) **Sepp R,** Csanády M. A hypertrophiás cardiomyopathia klinikai és molekuláris genetikája. *Orv Hetil* 1998; 139:1965-71.

V) **Sepp R,** Pálinkás A, Kertész E, Rampazzo A, Dongó Á, Jebelovszki É, Anastasakis A, Forster T, Danieli GA, Csanády M. Hypertrophiás cardiomyopathiát okozó génmutáció azonosítása a béta myozin nehéz lánc génben. Az első molekuláris genetikai analízissel igazolt magyar család leírása. *Cardiologica Hungarica* 2001, 1: 65-70.

VI) **Sepp R,** Jebelovszki É, Dongó Á, Rampazzo A, Pálinkás A, Piros Gy, Forster T, Danieli GA, Csanády M. Az első myozin kötő C fehérje génmutáció azonosítása magyar hypertrophiás cardiomyopathiás betegben. *Magyar Belorv Arch* 2001; 54:170-6.

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I) Sepp R, Pálinkás A, Kertész E, A Rampazzo, Dongó Á, Jebelovszki É, GA Danieli, Csanády M. A béta myozin nehéz lánc gén de novo mutációja malignus hypertrophiás cardiomyopathia hátterében. *Cardiologica Hungarica* 1999; 2(Supplementum): 18.

II) Sepp R, Pálinkás A, Kertész E, Dongó Á, Jebelovszki É, Anastasakis A, Miliou A, Theopistou A, Toutouzas P, Csanády M. Screening for mutations in the beta myosin heavy chain gene in patients with hypertrophic cardiomyopathy. *Hellenic Journal of Cardiology* 1999 Supplement B 40: 138.

III) Jebelovszki É, **Sepp R,** Dongó Á, Rampazzo A, Pálinkás A, Piros Gy, Forster T, Danieli GA, Csanády M. Az első myozin kötő C fehérje génmutáció azonosítása magyar hypertrophiás cardiomyopathiás betegben. *Cardiologica Hungarica* Supplementum 2000/3: 26.

IV) Sepp R, Melacini P, Rampazzo A, Barchitta A, Danieli GA. A myozin kötő C fehérje gén mutációja klinikailag tünetmentes lehet fiatal korban, hypertrophiás cardiomyopathiás betegekben. *Cardiologica Hungarica* Supplementum 2000/3: 16.

V) Sepp R, Borthaiser A, Jebelovszki É, Dongó Á, Rampazzo A, Pálinkás A, Forster T, Danieli GA, Csanády M. Myozin kötő C fehérje génmutáció hordozó, hypertrophiás cardiomyopathiás beteg családjának klinikai és genetikai analízise. *Cardiologica Hungarica* 2001; Supplementum (2001/2): 77.

VI) Sepp R, Borthaiser A, Jebelovszki É, Dongó Á, Rampazzo A, Kotta MC, Pálinkás A, Forster T, Anastasakis A, Danieli GA, Csanády M. Clinical and molecular genetic analysis of a family with hypertrophic cardiomyopathy caused by a myosin binding protein C gene mutation. *Acta Clin Croat* 2001, 40 (Supplement): 29.

VII) Dongó Á, **Sepp R,** Pálinkás A, Kertész E, Rampazzo A, Jebelovszki É, Kotta MC, Anastasakis A, Danieli GA, Csanády M. Identification of the first gene mutation in the beta myosin heavy chain gene causing hypertrophic cardiomyopathy in a Hungarian patient. *Acta Clin Croat* 2001, 40 (Supplement): 29.

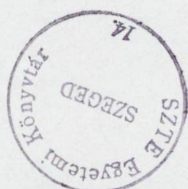
VIII) Sepp R, Borthaiser A, Jebelovszki É, Rampazzo A, Anastasakis A, Danieli GA, Csanády M. Identification of a novel stop codon mutation in the myosin binding protein C gene in a family with hypertrophic cardiomyopathy. *J Am Coll Card* 2002, 39 (Supplement B): 217B.

IX) Sepp R, Borthaiser A, Jebelovszki É, Dongó Á, Rampazzo A, Pálinkás A, Forster T, Danieli GA, Csanády M. Identification of a novel myosin binding protein C gene mutation causing hypertrophic cardiomyopathy. *J Mol Cell Card* 2002; 34:A57.

X) Sepp R, Dongó Á, Pálinkás A, Kertész E, Rampazzo A, Anastasakis A, Danieli GA, Csanády M. Early expression of a malignant phenotype of familial hypertrophic cardiomyopathy associated with an Arg719Gln beta myosin heavy chain gene mutation. *J Am Coll Card* 2002, 39 (Supplement B): 425B.

LIST OF ABBREVIATIONS

B-NHL	B-cell non-Hodgkin lymphoma
bp	base pair
DNA/RNA	deoxy/ribonucleic acid
HCM	hypertrophic cardiomyopathy
HPV	human papillomavirus
IgH	immunoglobulin heavy chain
ISH	in situ hybridization
MYBPC3	myosin binding protein C gene
MYH7	beta myosin heavy chain gene
PCR	polymerase chain reaction
SAM	systolic anterior motion (of the mitral valve)
SDS	sodium dodecyl sulphate
SSCP	single strand conformation polymorphism



INTRODUCTION

Molecular genetic diagnostics has become an ultimate tool to diagnose human diseases as it provides the only means to detect alterations at the level of genes. The diagnostics by molecular genetic methods can serve multiple purposes. One can detect disorders that are caused by the defective deoxyribonucleic acid (DNA) of the affected subjects (i.e. genetic diseases caused by mutations of the DNA) and therefore provide molecular diagnosis of a given disease. In cases of a known mutation, it is possible to identify mutation carriers in an affected family, in the majority of cases well before clinical symptoms develop. Molecular genetic methods are also able to detect exogenous DNA or RNA of disease causing infective organisms; the rearrangement of immunoglobulin genes proving monoclonality in certain lymphomas; or activation markers of a given condition. Furthermore, these investigations may provide valuable information on specific DNA variations, which may not cause human disease but rather predispose to certain disorders, or offer other essential information, such as subject identification in forensic applications.

DNA-based diagnostics were possible from the 70' but methods used in these investigations, such as Southern blotting or restriction fragment length polymorphism analysis, were lengthy, time and labour consuming. Furthermore, these methods usually required high molecular weight DNA obtained from relatively large amount of unfixed, fresh tissue, which limited the use of these techniques to certain circumstances. Obviously, these methods were not ideal for processing archive samples, which were stored in a fixed form.

In the late 80', a new method, the polymerase chain reaction (PCR) was developed,[1] which virtually revolutionised molecular biology. The reaction utilizes specific oligonucleotide primers and a thermostable DNA polymerase to amplify the region of interest of the template DNA in consecutive cycles. As all of the products from previous cycles can serve as templates for the next cycles, a chain reaction is initiated which produce virtually million copies of the original target sequence. PCR has rapidly become a basic procedure in all molecular biology laboratories and established itself as a diagnostic tool, as well. The technology quickly provided a powerful means for the rapid detection of infections, particularly those by viral pathogens,[2-5] of gene rearrangements in lymphoproliferative disorders,[6, 7] and of inherited diseases.[8] Furthermore, the technique could have served as an initial step for PCR based investigations.

An attractive feature of PCR is that, unlike other molecular biological procedures, high molecular weight DNA is not required for successful amplification. Since the turn of the century, pathologists commonly used formaldehyde-fixed, paraffin-embedded samples for tissue storage. Formilation of nucleic acids produces Schiff bases on free amino acid groups of nucleotides,[9-11] and exposure of nucleoproteins to formaldehyde results in the formation of cross-links between proteins and DNA.[12] This preserves tissue integrity and makes histological investigations possible. However, during the process the DNA content of the fixed tissue gets degraded. The degree of degradation is dependent of many factors, such as the duration of fixation,[2] the type of the fixative[2, 13] and some tissue related factors, such as the nuclease content of the tissue, the presence of necrosis, and certainly, autolysis. Therefore, although high molecular weight DNA is obtainable from fixed tissue,[14, 15] it is not possible in the majority of cases and the DNA obtained from these tissue sources are usually not suitable for traditional DNA analysis methods such as Southern blotting. As minute quantities of degraded DNA can serve as substrate for PCR, the method is ideally suited for a template extracted from formalin-fixed, paraffin-embedded archive material. The combined advantages of exquisite sensitivity and the ability to use routinely processed materials allow large scale, retrospective studies to be carried out. The method also made possible to examine DNA obtained from archaeological samples, such as mammoth,[16] Egyptian mummies,[17] or archaeological bone samples.[18]

Using archive material in molecular genetic investigations is clearly attractive as there are a vast amount of fixed and embedded tissue samples worldwide in pathology archives which represent an invaluable repository of all kind of human diseases, many of them with still unknown aetiology or a cause which was not known at that time. Furthermore, in some cases investigators have no biological sample taken in life (blood or tissue) and have only fixed tissue sample processed after death for further diagnostic work.

AIM OF THE WORK

The aim of the work was to investigate the use of routinely processed archive tissue samples in molecular genetic diagnostics.

We applied a two step approach to examine various aspects of the problem. In the first step we methodologically compared four rapid DNA extraction protocols from formalin-fixed,

paraffin-embedded material. In this comparative study the ability of the different methods to provide suitable DNA template for PCR was investigated on a wide variety of tissue sources and target sequences. In the second step we tested DNA templates extracted from archive tissue samples in molecular genetic diagnostic procedures. Since the two most frequently used molecular genetic diagnostic procedures in routine applications are virus identification and mutation detection, we choose these methodologies as test procedures.

We designed different projects for the above goals, aiming that the various studies would be coherent scientific projects in themselves and would provide valuable scientific information beyond methodological issues. Therefore we aimed:

1. To test rapid techniques for DNA extraction from routinely processed archive tissue for use in polymerase chain reaction.

The most widespread protocols for DNA extraction from formalin-fixed, paraffin-embedded tissues utilise shorter or longer proteolytic treatment, ranging from three hours to several days. The procedure is usually completed by organic solvent purification and ethanol precipitation.[2, 14, 15, 19, 20] An alternative procedure for tissue processing for PCR involves lysing of the cells by boiling in distilled water[3, 21, 22] or in a solution containing a chelating resin.[4, 5, 23] Other methods such as sodium dodecyl sulfate (SDS) incubation,[2, 22] sonication[24] and co-precipitation with Dextran Blue carrier[18] have also been described.

For a large scale, routine processing of archive material, DNA extraction should be simple and rapid, with no influence on the success of the following PCR. Moreover, as few steps as possible should be involved to minimise the possibility of contamination. The laborious and hazardous phenol-chloroform extraction should be particularly avoided.

Previous reports have focused mainly on the quantitative issues comparing DNA obtained from fresh or fixed tissues,[2, 5, 22] and have examined applicable methods separately[21, 23] or have compared them in the amplification only a few similar target sequences.[2, 4]

We performed a qualitative study, comparing several reported rapid DNA extraction procedures, each taking less than 4 hours. The ability of these methods to provide DNA suitable for PCR was investigated. For a more precise comparison, various sets of primers were chosen to amplify a single copy human gene, a viral sequence, and a rearranged immunoglobulin gene. Several practical aspects of PCR were also examined, taking into

consideration the size of the processed tissue, dewaxing, target sequence length and the reuse of extracts.

2. To screen archive lung carcinoma samples for the presence of human papillomaviruses by polymerase chain reaction.

Human papillomaviruses (HPV) are small double-stranded DNA viruses, which infect stratified epithelium. There are at least 80 different types, which can be divided into two subgroups: those infecting cutaneous surfaces and those infecting mucosal surfaces.[25, 26] While the viruses often cause benign proliferative lesions or warts, there are subgroups of viruses which cause premalignant and malignant lesions.[27] Malignant disease occurs most frequently in the genital tract and carcinoma of the cervix is far the most common cancer.

According to their potential role in the development of anogenital cancers (particularly in cervical carcinomas), HPV can be divided into high risk (e.g., HPV 16 and 18) and low risk (HPV 6 and 11) groups. Members of the former group may be involved in the promotion of carcinogenesis, and their DNA usually integrates into the host genome, whereas the latter types are found mainly in benign cervical lesions and show no integration.[28, 29]

Recently, HPV DNA has also been detected in squamous cell mucosal malignancies apart from the anogenital region. Mainly high risk type viruses were found in carcinomas of the oral[30] and nasal cavity,[31] the male urethra,[32] the urinary bladder,[33] and the oesophagus.[34, 35] With regard to the respiratory tract, HPV 16 was detected in laryngeal carcinomas,[36] and HPV 16 and 11 was found to be associated with laryngeal papillomas.[37] The latter, histologically benign proliferations may spread into the inferior airways or may occur alone in the trachea and bronchi. In addition, malignant transformation was reported in several cases, where squamous cell laryngeal[38] and bronchial cancers[39-42] originated from benign airway papillomas containing HPV. Early studies on lung cancers not preceded by known respiratory tract papillomas also found variable degree of HPV presence,[43-46] using in situ hybridization (ISH) in most of the investigations.

Our aim was to examine the occurrence of HPV in squamous and large cell (which may also have squamous cell origin) lung neoplasm of fixed, archive tissue samples. We searched for high risk and low risk viruses applying PCR, which offers an accurate and rapid means of viral detection with a higher sensitivity than ISH in previous studies.[28]

3. To search for disease causing mutations in archive samples of patients with hypertrophic cardiomyopathy.

Hypertrophic cardiomyopathy (HCM) is defined as a heart muscle disease of unknown origin, which is characterised by asymmetric hypertrophy of the left ventricle, which typically involves the septum more than the free wall.[47] Typically, the left ventricular volume is normal or reduced. Epidemiology data put the prevalence of HCM between 3.2-33/100.000 but recent data suggest that HCM is more common than it was previously thought; approximately 1/500-1000.[48] Patients may be clinically asymptomatic or affected by varying degrees of dyspnoea, chest pain, palpitation or syncope. Rhythm disturbances are common and there is an increased risk of sudden cardiac death.

Recent research in molecular genetics has revealed that HCM is a genetic disorder transmitted as an autosomal dominant trait. To date, mutations in twelve genes have been found in patients with HCM. The majority of these genes encode for proteins of the sarcomere, i.e. the beta myosin heavy chain gene[49] (*MYH7*, 14q11), the alpha tropomyosin gene[50] (*TPM1*, 15q22), the troponin T,[50] I[51] and C[52] genes (*TNNT2*, 1q3; *TNNI3*, 19p11; *TNNC1*, 3p21-p14), the myosin binding protein C gene[53, 54] (*MYBPC3*, 11p11.2), the essential (*MYL3*, 3p21.31) and the regulatory myosin light chain genes[55] (*MYL2*, 12q24.21), the cardiac actin gene[56] (*ACTC*, 15q12) and the titin gene[57] (*TTN*, 2q35), while some of these genes code for other proteins, i.e. the gamma2 regulatory subunit of the AMP-activated protein kinase gene[58] (*PRKAG2*, 7q36) and cardiac muscle LIM protein gene[59] (*CLP*, 11p15). As the majority of the above genes encode for proteins of the cardiac sarcomere, HCM has been reclassified as the disease of the sarcomere.[50]

Among the HCM genes, the beta myosin heavy chain gene has been shown to be affected most frequently, accounting for approximately one third of HCM cases. The mutations in the gene are almost exclusively missense mutations of the first 23 exons, affecting the globular head of the myosin molecule.[60] Based on limited number of observations, it has been suggested that some mutations are associated with a malignant clinical phenotype with severe symptoms and early occurrence of sudden cardiac death, while other mutations do not alter significantly the natural course of the disease.[61]

The other most frequently affected HCM gene is the myosin binding protein C gene, which accounts for approximately 30-35% of all HCM cases.[60] Most of the mutations in the myosin

binding protein C gene, unlike other HCM mutations, are "splice site" mutations, which lead to a truncated protein. The truncation affects the distal part of the protein, which includes the essential myosin and titin binding domain. The phenotype caused by a myosin binding protein C mutation usually shows an age-dependent penetrance, mild left ventricular hypertrophy and a benign clinical course.

We aimed to detect disease causing mutations in archive tissue samples of patients with hypertrophic cardiomyopathy. As sudden cardiac death could be the first manifestation of the disease, a tissue sample taken at the time of an autopsy is the only available material in some of the cases.

MATERIALS AND METHODS

Materials

1. For testing rapid techniques from routinely processed archive tissue for use in polymerase chain reaction, the following material was used: Blocks of formalin-fixed, paraffin wax embedded archive tissues were selected from the files of the Department of Pathology, Kagawa Medical School, Japan. Normal sized (average surface dimensions 16x18 mm) and biopsy specimen sized (average surface dimensions: 1.7x2 mm) samples were collected and divided into the following groups:

1) Twelve blocks of normal sized and six blocks of biopsy specimen sized material, randomly chosen from various tissues; they represented neoplastic, inflammatory, or hyperplastic processes of the skin, soft tissue, tonsils, oesophagus, duodenum, colon, pancreas, lung, kidney, urinary bladder, meninges, ovary, cervix, normal placenta, and endometrium;

2) Twelve blocks of normal sized cervical cancer samples and additional six blocks of cervical biopsy specimens (diagnosed as cancer or severe dysplasia), known to harbour human papillomavirus type 16 (HPV 16) DNA by the authors' previous PCR assays;

3) Six blocks of normal sized non-Hodgkin's B cell lymphoma samples (B-NHL) and an additional three blocks of B-NHL biopsy specimens, known to have detectable immunoglobulin heavy chain (IgH) gene rearrangement according to the authors' previous PCR results.

The material was processed between 1988 and 1993. Fixation was in neutral formalin, and the fixation time varied from 24 to 48 hours. Processing was done by standard methods: dehydration in graded alcohols; clearing in xylene and embedding in paraffin wax.

2. To screen archive lung carcinoma samples for the presence of human papillomaviruses by polymerase chain reaction, the following material was used: Tissue samples with previously diagnosed squamous (n=40) and large cell (n=7) lung carcinomas were examined. All were primary neoplasms resected between 1990 and 1993 at the Second Department of Surgery of Kagawa Medical School, Japan. None of the patients were known to have previous airway papillomas. One formalin-fixed, paraffin-embedded tissue sample of tumour from each case was investigated. The squamous cell cancers were diagnosed as well, moderately, and poorly differentiated in 5, 18 and 17 cases, respectively. Koilocytic atypia, which is thought to

be a histological marker of HPV infection, was detected in six cases (all squamous cell carcinomas) and was diagnosed by the presence of cells with pleiomorph, hyperchromatic nuclei and sharply marginated perinuclear clearing.

Positive controls comprised HPV 16 DNA cloned into pUC19 plasmid (Genemed, San Francisco, CA), DNA extracted from HPV 16-carrier cervical cancer, and DNA from HPV 6/11 carrier perianal condyloma acuminatum. All positive controls were typed by previous PCR assays using other type-specific HPV primers. The same DNAs were used as negative controls in cross reactions, and distilled water was used as a reagent control.

3. To search for disease causing mutations in archive samples of patients with hypertrophic cardiomyopathy, the following material was used: Two patients who died of hypertrophic cardiomyopathy were investigated.

Case history, case No 1.

The Caucasian (Hungarian) proband was first hospitalized in 1978 at the age of 12 because of the suspicion of a ventricular septal defect. ECG at that time showed sinus rhythm, left ventricular hypertrophy with repolarisation changes. Cardiac catheterisation ruled out the suspected ventricular septal defect, and subsequent echocardiography showed asymmetric septal hypertrophy. She was put on beta-blocker therapy and remained in stabile clinical condition with occasional symptoms of palpitations, effort dyspnoea and dizziness. Syncope, thromboembolism or cardiac decompensation did not occur. Holter monitoring showed 1% ventricular premature beats, but no sustained or non-sustained ventricular tachycardia. On last follow up, at the age of 31, her ECG displayed sinus rhythm, non-specific intraventricular conduction block with secondary repolarisation changes and ST elevation in leads V1-5. Echocardiography showed typical asymmetric septal hypertrophy (interventricular septum thickness: 21 mm, posterior wall: 9 mm), mildly dilated left atrium, normal left ventricular parameters and function and abnormal relaxation (E/A: 48/68). No outflow tract gradient or systolic anterior motion of the mitral valve (SAM) was present. One year later, at the age of 32, she died suddenly while she was swimming in the swimming pool.

Clinical screening of the proband's family members indicated that neither the proband's patients nor her siblings were affected (see family tree in Figure 7). One of the proband's two sons, however, was known to have HCM since the age of 2. ECG at that time showed sinus rhythm, biventricular hypertrophy with strain. Echocardiography revealed asymmetric hypertrophy with a septal thickness

of 21 mm (posterior wall 7 mm), altered relaxation and SAM. He was put on beta-blocker therapy. During follow up he always had a decreased exercise tolerance, with effort dyspnoea, but he never had syncope, chest pain or thromboembolism. He was hospitalized several times because of bronchitis and bronchopneumonia. On last follow up (at the age of 8) he had sinus rhythm, left axis deviation, biatrial strain, left ventricular hypertrophy and pathological Q waves in inferior leads. Echocardiography showed left ventricular hypertrophy in all of the left ventricular segments except the inferior and posterior wall and hypertrophy of the right ventricular outflow tract. The hypertrophy was the most pronounced at the middle of the septum (22 mm). Left atrial enlargement, mild left ventricular dilatation and presumably pseudonormal diastolic function (E/A: 50/33) was also present. Holter monitoring was normal. Exercise test proved decreased exercise capacity and hypotensive blood pressure response.

Case history, case No 2.

The proband's symptoms occurred at the age of 43; one year later she presented with symptoms of fatigue and swollen legs. ECG showed sinus rhythm, left atrial overload, with ST-T changes in lateral and inferior leads. Echocardiography revealed asymmetric septal hypertrophy (interventricular septum: 32 mm, posterior wall: 9 mm), with mildly dilated left atria (45 mm), small left ventricle (left ventricular end diastolic diameter: 39 mm, left ventricular end systolic diameter: 20 mm) and preserved left ventricular systolic function. Neither left ventricular outflow tract obstruction nor SAM was present. During the 6 year follow up she remained asymptomatic on beta blocker/retard verapamil therapy; she was free of syncopal episodes and major ventricular arrhythmias. Echocardiographic follow up showed a decrease of the septal thickness and the increase of the left atrial and left ventricular parameters, but no left ventricular dilatation has occurred. She died at the age of 49 after having syncope at home. Autopsy revealed a hypertrophied heart, weighing 490 grams, and old myocardial scars with intramural thrombi were noted. The left ventricular thrombosis led to embolism to the brain, to the right kidney and to the liver. Histology showed "myofiber disarray", a histological hallmark of hypertrophic cardiomyopathy; myocyte hypertrophy, disseminated fibrosis and small vessel disease.

According to her family history her mother (I:2, see family tree in Figure 10, upper panel) died at the age of 55 because of cervical cancer and her father died (I:1) at the age of 66 because of some "heart disease". On family screening, three of her children (III:1, III:2, III:3), her brother (II:2) and two of her nieces (III:4, III:5) were available, all of them were free of cardiac signs or symptoms, and exhibited normal ECG and echocardiogram.

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Blocks of formalin-fixed, paraffin-embedded tissue of heart; spleen and liver, routinely processed at the time of autopsy were selected for DNA extraction. From each block a representative histological slide was cut and stained with haematoxylin-eosin to avoid blocks with extreme tissue degradation and autolysis. In one case we had also a blood sample taken in life which was also processed.

Methods

1. For testing rapid techniques for DNA extraction from routinely processed archive tissue for use in polymerase chain reaction we compared the following methods:

DNA extraction

From normal sized tissues, a single 5 μm section (average collected volume 1.44 mm^3) and from biopsy specimen sized tissues a single 10 μm section (average collected volume 0.034 mm^3) was cut and processed according to one of the following four methods (Methods A-D).

Method A: The section was resuspended in 100 μl distilled water (50 μl for biopsy sized material), with or without dewaxing, and was boiled for 20 minutes (modified from the method of Lench *et al*[22]). Dewaxing was carried out by two washes in xylene at 60°C for 20 minutes, rehydration in absolute ethanol, and desiccation.

Method B: This method is similar to Method A, but the section was resuspended in 5% Chelex-100 resin solution (Bio Rad Laboratories, Hercules, California, USA) and was boiled for 20 minutes.

Method C: After dewaxing and rehydration, the tissue section was resuspended in 100 μl (50 μl for biopsy sized specimen) of digestion buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 0.5% Tween 20, pH 9 at 25°C) containing 200 μg/ml proteinase K (Merck, Darmstadt, Germany) and was incubated at 55°C for 3 hours (or at 37°C overnight).[62] The proteinase K was inactivated by boiling for eight minutes.

Method D: This method is similar to Method C, but after digestion, Chelex-100 was added to a final concentration of 5%, and the mixture was boiled for eight minutes (modified from the method of Gill *et al*[63]).

After boiling, in the case of all methods, the tubes were chilled on ice, and were centrifuged for five minutes at 18 000 x g. A 5 µl aliquot of the supernatant (10 µl for biopsy specimen sized tissue) was used as template.

Oligonucleotide primers

The sequences, target regions and other characteristics of the primers are shown in Appendix 1. Three types of primer sets were used: 1) one for various exons of the p53 gene, which represents a single copy human gene, on DNA extracted from the randomly chosen tissues; 2) a second set of primers for the open reading frame region E6 of HPV 16 on DNA extracted from the cervical samples; 3) and a third set of primers for the IgH gene rearrangement on DNA extracted from B cell lymphomas. Oligonucleotides were synthesised on a Cyclone Plus automated DNA synthesiser (Millipore, Bedford, Massachusetts, USA).

PCR

The PCR was carried out in a Perkin Elmer Cetus Thermal Cycler (Perkin-Elmer, Norwalk, Connecticut, USA). The reaction mixture (in 50 µl or 25 µl total volume) contained 10-20 pmol of primers, 1x PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 9 at 25°C, 3 mM $MgCl_2$), 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 0.625-1.25 U of *Taq* polymerase (Pharmacia Biotech, Milwaukee, Wisconsin, USA), and the template. The reaction mixture was overlaid with a drop of light mineral oil (Sigma, St. Louis, Missouri, USA).

Each PCR cycle consisted of a denaturation step at 94°C for 30 seconds, primer annealing at 60°C (55°C for H1 and H2 primers) for 30 seconds, and extension at 72°C for 1 minute. An initial denaturation step was performed at 94°C for 2 minutes, and in the final cycle the extension step was elongated to 5 minutes at 72°C. Samples were amplified through 35 (for normal sized samples) or 40 cycles (biopsy samples). For biopsy specimen sized B cell lymphoma samples, semi-nested PCR was performed: a first round of 30 cycles with primers

Fr3A and LJH; and a second round of 35 cycles with Fr3A and VLJH, with 1 µl of the product of the first round of the PCR as the template.

Positive and negative controls were included in all runs. The positive and negative controls were as follows: HPV 16 positive DNA (Genemed, San Francisco, California, USA) and DNA extracted form human T cell lymphotrophic virus type I (HTLV-I) carrier cell line, respectively for the HPV 16 PCR; high molecular weight DNA extracted from fresh frozen human lung and distilled water, respectively, for the p53 PCR; and DNA extracted from paraffin wax embedded B cell and T cell lymphoma tissues, respectively, for the IgH PCR.

Sterile materials were used throughout, with strict precautions to avoid contamination or product carry-over. DNA extraction, PCR, and electrophoresis were performed in separate laboratories.

Gel analysis

The quality of the template DNA was determined by running the samples on 1% agarose gel (Wako Chemicals, Osaka, Japan) stained with ethidium bromide. A 10 µl aliquot of the amplification products were analysed by electrophoresis through 3% NuSieve GTG agarose (FMC Bioproducts, Rockland, Maine, USA), followed by ethidium bromide staining visualised by ultra violet light transilluminator. Bands of appropriate size were identified by comparison with DNA marker of known size.

2. To screen archive lung carcinoma samples for the presence of human papillomaviruses by polymerase chain reaction, the following methods were used:

DNA preparation

DNA extraction was performed with modifications from the above protocols, as it follows (Method E). Briefly, an average of three 10 µm sections were cut from each block, dewaxed in xylene, and rehydrated in absolute ethanol. After desiccation, the tissue was resuspended in 200 µl digestion buffer (100 mM sodium chloride, 10 mM Tris-hydrochloric acid, 5 mM EDTA, 0.5% SDS, pH 8.4) containing 200 µg/ml Proteinase K (Boehringer Mannheim, Mannheim, Germany) and was incubated at 37°C for 4 days. The DNA was extracted with phenol-chloroform-isoamyl alcohol, precipitated in absolute ethanol, and redissolved in TE

buffer (10 mM Tris-1 mM EDTA, pH 8). The DNA content was quantitated by a GeneQuant DNA/RNA Calculator (Pharmacia LKB, Cambridge, England). 300 ng DNA was used as template in each PCR reactions.

Oligonucleotide primers

Consensus primers were used designed by Fujinaga *et al*[64] (see Appendix 1). The sense primers, pU-1M and pU-31B, are located in the middle of the open reading frame E6 on the HPV genome, and the anti-sense primer, pU-2R, is located in the middle of the open reading frame E7. The primers pU-1M and pU-2R detect HPV 16, 18, 31, 33, 52b, and 58 (high risk types), whereas pU-31B and pU-2R detect HPV 6 and 11 (low risk types). The primers pU-1M/pU-2R amplify a fragment of 231-268 bp, whereas primers pU-31B/pU2R yield a fragment of 228 bp.

PCR and gel analysis

The PCR reactions (with an annealing temperature of 55°C) and gel analysis were performed as described above.

3. To search for disease causing mutations in archive samples of patients with hypertrophic cardiomyopathy, the following methods were used:

DNA preparation

DNA extraction was performed according to Method E (i.e. Proteinase K digestion followed by phenol-chloroform extraction and ethanol precipitation).

Oligonucleotide primers

The sequences of the primers for amplifying the beta myosin heavy chain gene (*MYH7*) exon 19 and the myosin binding protein C gene (*MYBPC3*) exon 33 are given in Appendix 1. All primer pairs are intronic, flanking one exon of the gene.

PCR

The PCR reaction was performed as described above, with annealing temperatures of 52-62°C, optimised beforehand.

Mutation analysis

PCR products were analysed for the presence of sequence variations by the 'single strand conformation polymorphism' (SSCP) method. A 5 µl aliquot of the PCR product was mixed with denaturing buffer (containing 80% formamide, 10 mM EDTA, pH 8.0, 1 mg/ml xylene cyanole and 1 mg/ml bromophenol blue), denatured at 95°C for 10 minutes, and was chilled on ice for 5 minutes. The denatured samples were loaded on 10% non-denaturing polyacrylamide gels and were run at 5-25°C on constant voltage of 200 V for 16-20 hours. The gels were stained with silver using standard methods. Samples showing altered migration patterns were direct sequenced on an automated sequencer (Applied Biosystems).

RESULTS

1. Rapid techniques for DNA extraction from routinely processed archive tissues produce comparable templates for polymerase chain reaction.

The efficiency of DNA extraction methods A-B-C-D was tested by PCR reactions on DNA extracted from various sample types. The overall results obtained by these PCRs using the different primer sets are summarized in Table 1.

	Method			
Material	**A**	**B**	**C**	**D**
Normal sized				
various samples	12/12	12/12	12/12	12/12
cervix samples	12/12	12/12	12/12	12/12
B cell lymphoma samples	5*/6	6/6	6/6	6/6
Biopsy specimen sized				
various samples	4†/6	6/6	6/6	6/6
cervix samples	6/6	6/6	6/6	6/6
B cell lymphoma samples	3/3	3/3	3/3	3/3
Total	**42‡/45**	**45/45**	**45/45**	**45/45**

Table 1. PCR results on different materials prepared by Methods A-D (numbers shown are positive cases/total cases). *Fainter bands in four cases; †fainter bands in two cases; ‡fainter bands in six cases

P53 gene amplification on DNA templates extracted from various randomly chosen tissues

A 166 bp fragment of exon 8 or a 99 bp fragment of exon 4 of the p53 gene were amplified. All of the templates prepared from normal sized tissues by different methods produced a single specific amplification band with approximately the same intensity (Figure 1, lanes 1a-1d). However, two of the biopsy specimen sized samples (33% of the specimens) prepared by the water boiling method (Method A) failed to amplify, and in two other cases (50% of the successful amplifications) the bands were much fainter. Biopsy samples prepared by Chelex boiling (Method B), proteinase K digestion (Method C) and proteinase K digestion-Chelex boiling (Method D) provided uniformly intense bands (Figure 1, lanes 2a-2d).

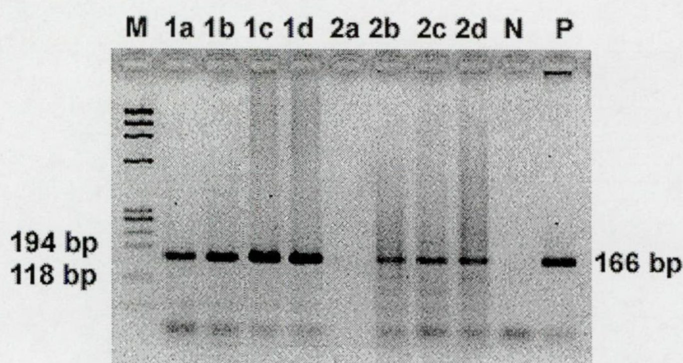

Figure 1. 3% agarose electrophoresis gel of the PCR products, showing the amplification of a 166 bp fragment of exon 8 of the p53 gene from DNA extracted from normal and biopsy specimen sized paraffin wax embedded tissue by different methods. Marker: ΦX174 DNA Hae III digest. 1a-d: normal sized tissue sample of colonic adenocarcinoma processed by Method A (1a), B (1b), C (1c), and D (1d). 2 a-d: biopsy specimen of duodenal polyp processed by Methods A (2a), B (2b), C (2c), and D (2d). N: negative control (distilled water instead of template). P: positive control (high molecular weight DNA as template, extracted from fresh frozen human lung). The sizes of the molecular weight marker and the specific band (in bp) are shown to the left and right, respectively. An inverse of the gel image is shown.

HPV 16 amplification on templates extracted from cervical samples

A 110 bp fragment of the open reading frame region E6 of HPV 16 was amplified. All of the samples prepared by the different methods from both normal and biopsy specimen sized tissues were successfully amplified (data not shown).

Detection of immunoglobulin gene rearrangement on templates extracted from B cell lymphoma samples

Depending on the given rearrangement of particular clones of B cell lymphomas, an 80-120 bp fragment was amplified. One normal sized lymphoma specimen (17% of the normal sized lymphomas) prepared by the water boiling method (Method A) failed to amplify and in four other cases (80% of the successful amplifications) much fainter bands were observed. DNA extracted by the other three methods produced intense bands of appropriate size in all cases (Figure 2). Templates extracted from the three biopsy specimens investigated by semi-nested PCR showed similar strong bands, regardless of the method used for extraction.

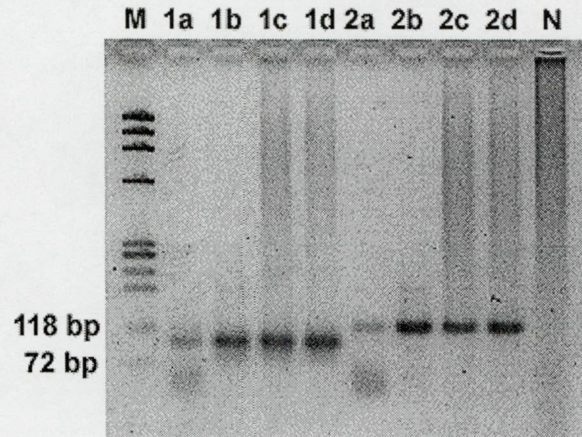

Figure 2. 3% agarose electrophoresis gel of the PCR products, showing amplification of an ~100-120 bp fragment of a rearranged immunoglobulin heavy chain gene from DNA extracted from two normal sized B cell lymphoma samples by different methods. M: ΦX174 DNA Hae III digest. 1a-d: parotidal B cell lymphoma processed by Methods A (1a), B (1b), C (1c), and D (1d). 2 a-d: intestinal B cell lymphoma processed by Methods A (2a), B (2b), C (2c), and D (2d). N: negative control (DNA extracted from a T cell lymphoma sample as a template). The sizes of the molecular weight marker bands (in bp) are shown to the left. An inverse of the gel image is shown.

Analysis of previous dewaxing

In order to reduce further the number of steps involved in the extraction, dewaxing and rehydration steps were omitted from the water boiling and Chelex boiling protocols. We noted no difference in the success of amplifications using these extracts (data not shown).

Analysis of template quality

The DNA extracted by the different methods was analysed on 1% agarose gel. There was evidence of extreme DNA degradation in templates prepared by water boiling (Method A), with the bulk between 100 and 200 bp. The DNA obtained by Chelex boiling (Method B) was better preserved, with a distribution in the 100-600 bp range. The proteinease K digestion methods (Methods C and D) provided DNA of increased molecular weight with a range from 100 to over 10 000 bp, with the bulk between 100 and 4000 bp (Figure 3).

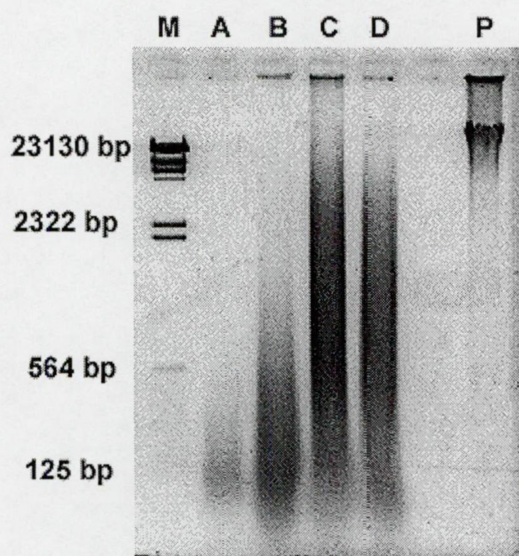

Figure 3. 1% agarose electrophoresis gel of DNA extracted from a normal sized oesophageal carcinoma sample by different methods. M: λ DNA Hind III digest; A: boiled in distilled water (Method A); B: boiled in 5% Chelex solution (Method B); proteinase K digested (Method C); proteinase K digested and boiled in 5% Chelex solution (Method D); P: positive control (high molecular weight DNA extracted from fresh frozen human lung). The sizes of the molecular weight marker bands (in bp) are shown to the left. An inverse of the gel image is shown.

Analysis of the target sequence length

Combining forward and reverse primers, homologous to consecutive exons of the p53 gene, PCR products of gradually increasing length were amplified. Primers E51 and E52 (flanking exon 5) amplified a 214 bp PCR product, while primers E51 and E62 (flanking exon 5 and 6), primers E71 and E82 (flanking exon 7 and 8) and E61 and E72 (flanking exon 6 and 7) amplified 408, 647, and 983 bp PCR products, respectively. Two normal sized samples were analysed by these primer combinations.

Proteinase K digested and Proteinase K digested-Chelex boiled templates were successfully amplified up to 983 bp (data not shown), although with an accumulation of non-specific products owing to uncompleted amplifications. Chelex boiled material showed a strong signal for 214 and 408 bp products, and still produced a weak band on the 647 bp product. On DNA templates originated from water boiled material only 214 and 408 bp products were amplified (Figure 4).

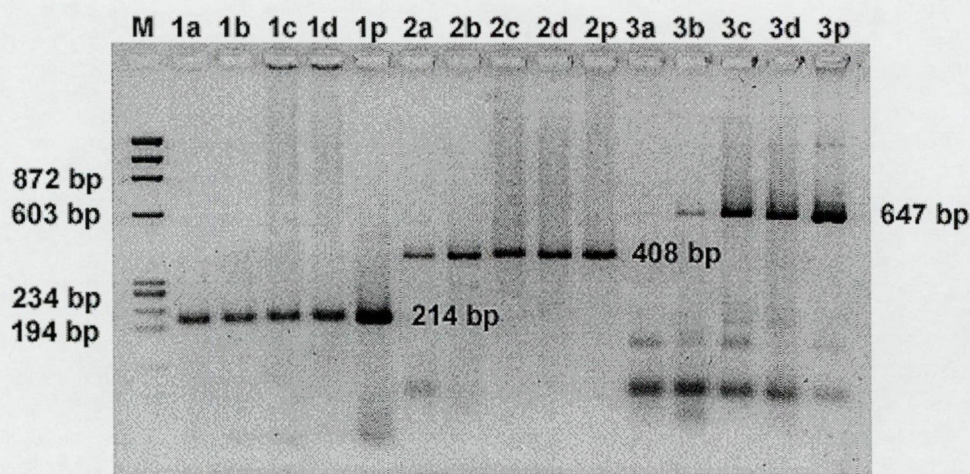

Figure 4. 2.5% agarose electrophoresis gel of PCR products showing amplification of exon 5, exon 5-6, and exon 7-8 of the p53 gene from DNA extracted from a normal sized cervical cancer sample by different methods. M: ΦX174 DNA Hae III digest. 1 a-d: amplification of 214 bp fragment from extracts prepared by Method A (1a), B (1b), C (1c) and D (1d); 2 a-d: amplification of 408 bp fragment from extracts prepared by Method A (2a), B (2b), C (2c) and D (2d); 3 a-d: amplification of 647 bp fragment from extracts prepared by Method A (3a), B (3b), C (3c) and D (3d); 1p, 2p, 3p: positive controls (high molecular weight DNA as template, extracted from fresh frozen human lung). The sizes of the molecular weight marker and the specific band (in bp) are shown to the left and right, respectively. An inverse of the gel image is shown.

Analysis of the reuse of templates

After three month of storage at -20°C, two samples from each group prepared by the four methods were successfully reamplified for their specific targets. However, 408 bp targets produced weaker bands than freshly prepared samples, and amplification for the 647 bp fragment was successful only in proteinase K digested and proteinase K digested-Chelex boiled samples (data not shown).

2. Human papillomaviruses were not found in archive samples of squamous and large cell lung cancers by polymerase chain reaction.

In the lung carcinoma samples analysed, neither high risk (HPV 16, 18, 31, 33, 52b, and 58) nor low risk (HPV 6 and 11) type viruses were detected in any sample (Figure 5). Positive controls always gave strong positivity and negative controls were consistently negative.

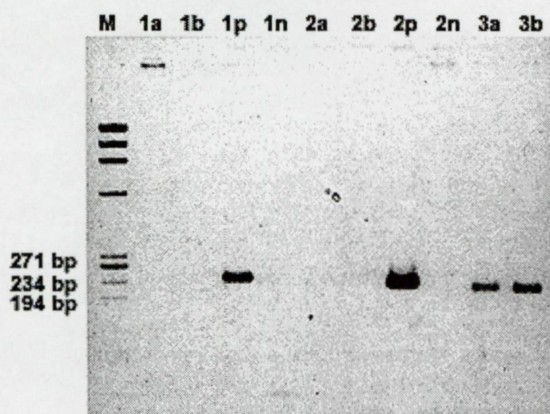

Figure 5. 2.5% agarose electrophoresis gel showing the results of PCR on a squamous and a large cell lung neoplasm. Amplifications with primers specific for high risk HPV showed no positivity in the squamous (1a) and the large cell (1b) carcinoma samples, but produced the expected 238 bp fragment in the positive control (1p). Amplifications with primers specific for low risk HPV showed no positivity in the squamous (2a) and large cell (2b) carcinoma samples, but produced the expected 228 bp fragment in the positive controls (2p). The negative controls for high risk and low risk HPV are shown in 1n and 2n, respectively. The same lung cancer samples showed amplification for the 214 bp fragment of exon 5 of the p53 gene (3a-b). M: molecular weight marker (ΦX174 DNA Hae III digest). The sizes of the molecular weight marker and the specific band (in bp) are shown to the left. An inverse of the gel image is shown.

To ensure that sufficient DNA was present in the reaction mixture and the PCR was not inhibited, we tested our samples with primers of a single copy human gene, and we were able to amplify a 214 bp fragment of exon 5 of p53 gene in all of our samples. Since the PCR parameters we used were slightly modified from those described by Fujinaga *et al*,[64] we compared the original and this method on the positive controls. No difference was found concerning the presence and the intensity of the specific product.

3. Detection of an Arg719Gln mutation of the beta myosin heavy chain gene and an Arg1226Stop mutation of the myosin binding protein C gene in archive tissue samples of patients with hypertrophic cardiomyopathy.

Genetic analysis, case No 1.

On SSCP analysis of the proband's sample, exon 19 of the beta myosin heavy chain gene (*MYH7*) showed a band with altered migration pattern. This product was sequenced and sequencing revealed a G to A transition at position 13463 (Figure 6). A corresponding C to T transition was also present in the identical position in the anti-sense strand.

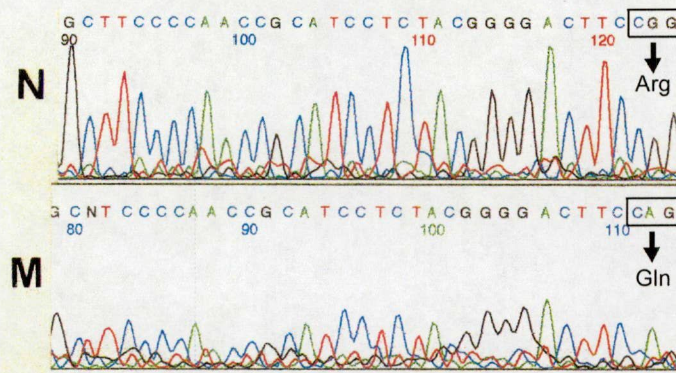

Figure 6. Sequence analysis of exon 19 of the beta myosin heavy chain in the patient's (M) and in a control sample (N). Sequencing reveals a G-A transition at position 13463 which changes the code CGG (coding for arginine) to CAG (coding for glutamine) at codon 719.

The mutation causes an amino acid change of arginine to glutamine at codon 719 (Arg719Gln). The mutation abolishes a restriction site for MspI; which prevented the digestion of the wild type 210 bp PCR product into two fragments (which are 147 and 63 bp, respectively) in the mutated allele (Figure 8).

On screening the proband's family members for the presence of the mutation (Figure 7) we found that only the proband's clinically affected son carried the mutation (Figure 8).

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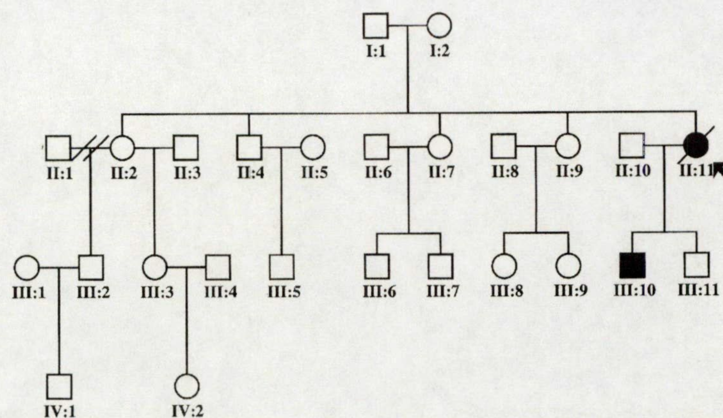

Figure 7. Family tree of the investigated family. Circles represent females, squares represent males. Clinically affected family members are filled, unaffected are clear. The index patient (II:11) is marked by arrow.

None of her parents had the mutation (paternity has been proven by microsatellite analysis), suggesting that the mutation may have arisen 'de novo'. The mutation was not present in 100 chromosomes from geographically identical normal population. Both the proband and the affected son carried a normal allele indicating that the mutation was present in heterozygous form.

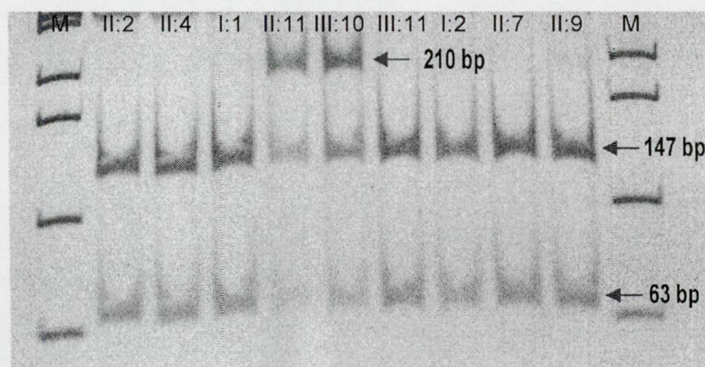

Figure 8. MspI restriction fragment length polymorphism analysis of exon 19 of the beta myosin heavy chain gene in the affected family. In unaffected family members the original 210 bp PCR fragment is cleaved to a 147 bp and a 63 bp fragment. In affected family members (II:11, III: 10, see family tree), the mutation abolishes the restriction site on the mutated allele, and cannot be cut. The mutation present in a heterozygous form, as a normal, cut allele is also present in the affected subjects. Only the affected mother (II: 11) and affected son (III:10) carries the mutation, neither her father (I:1), nor her mother (I:2). M: 100 bp ladder as molecular weight marker. The numbering above the lanes represents identification in the family tree.

Genetic analysis, case No 2.

A band with abnormal mobility in exon 33 of the myosin binding protein C gene (*MYBPC3*) was identified by SSCP analysis. Direct sequencing of the PCR product revealed a cytosine to timine change in nucleotide position 19489 (C19498T), which were also proven by sequencing the anti-sense strand (Figure 9). The mutation caused a change of glutamine (CAG) for a nonsense stop codon (TAG) at codon 1226 (Gln1226Stop). The mutation was present in heterozygous form in the patient, harbouring a normal and a mutated allele. No such base change was detected by screening 100 normal chromosomes.

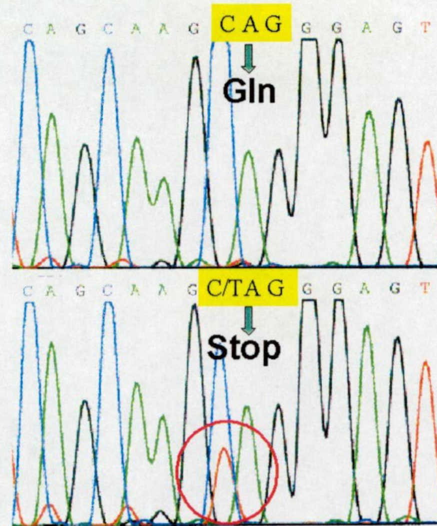

Figure 9. Sequence analysis of exon 33 of the myosin binding protein C gene in the patient's (lower panel) and in a control sample (upper panel). Sequencing reveals a C-T transition at position 19489 which changes the code CAG (coding for glutamine) to TAG (coding for a stop codon) at codon 1226.

By mutation screening of the family members we found that two of the patient's children were affected, while other family members were non-carriers (Figure 10).

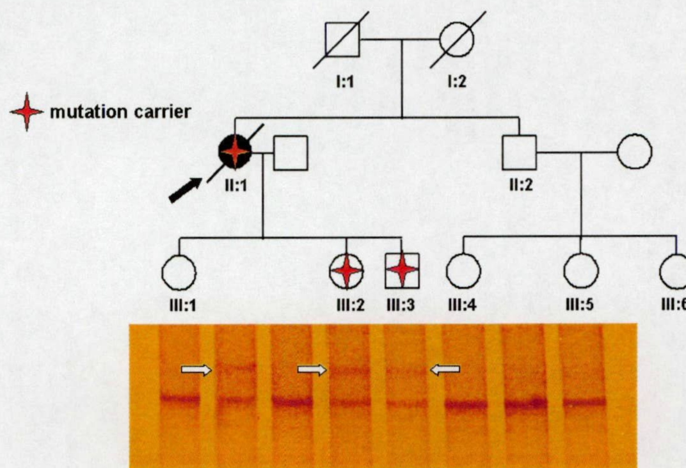

Figure 10. Upper panel: Family tree of the affected family. Circles represents females, squares represents males. Clinically affected family members are filled, unaffected are clear. The index patient (II:1) is marked by arrow. Lower panel: mutation analysis of family members by SSCP, lanes represents family members above the gel. Family members III:2 and III:3 show the same SSCP pattern (arrows) as that of the proband (II:1).

Both mutation carrier siblings are free of signs or symptoms of the disease, have normal ECG and echocardiogram (apart of the presence of mitral valve prolapse).

DISCUSSION

Formalin has been used as a fixative in pathology for more then one hundred years. The criteria employed by pathologists for the diagnosis of many diseases have been established in formalin-fixed, paraffin-embedded tissue sections stained with haematoxylin and eosin. During more than a century of diagnostic practice, a large number of archive paraffin-embedded tissue banks have been established worldwide. These tissue banks form invaluable resources for examining human pathologies of various origins and we have to find the ways to use these sources for molecular biological investigations.

The process of fixation preserves histological integrity of tissues, but degrades the high-molecular weight DNA content. The reason why formalin-fixed tissue undergoes degradation is not exactly known. Possibilities include insufficient neutralisation of the formalin, eventually resulting in acidic depurination. Acid is known to depurinate DNA and would destroy the DNA template. It was also shown that the type of fixative is also important for the recovery of DNA from fixed tissues.[2, 13] Specimens fixed in picric acid (Bouin's reagent) or mercuric chloride (Zenker's fixative, B5) based fixatives provide less yield and more degraded DNA. Ethanol based fixatives (Carnoy's) result in a much better preservation of DNA in the tissue, allowing the extraction of high molecular weight DNA and providing quantitatively much higher yields. Alternatively it may not be the formalin fixation itself but the specific type of tissue, its necrotic stage and nuclease content that are more important in the recovery of intact DNA.

In our work we aimed to investigate the use of routinely processed archive material in the use of molecular genetic diagnostics. We developed three different, independent scientific projects to analyse different aspects of DNA extraction from archive tissues and the applicability of archive samples for detection human viral pathogens and disease causing human mutations were examined.

1. Testing rapid techniques for DNA extraction from routinely processed archive tissue for use in polymerase chain reaction.

Although formalin-fixed tissues can also provide high molecular weight DNA,[14, 19] suitable for complex molecular biological investigations, the procedure requires lengthy digestion and purification steps, which would be difficult to perform on a large scale. The major attraction of

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the PCR, for research and diagnostic purposes in pathology, is its ability to work on poor quality DNA samples obtained by simple methods of extraction.

In our work we examined four different rapid techniques of DNA extraction, from paraffin embedded tissues. Using these techniques PCR results can be obtained within 4-8 hours. DNA produced by the simplest water boiling method failed to be amplified for specific sequences in 7% of the total cases. In addition, in 14% of the positive samples the signal was much weaker than in cases using other methods. Several investigators have also reported a variable degree of failure using this technique.[4, 21, 23] It seems likely that the cause of failure is the absence of a detectable amount and the low quality of template DNA. Theoretically, the amount of the released target DNA can be small if the size of the processed tissue is small (a biopsy specimen) or if only a portion of the processed material carries the target sequence (monoclonal B cells with a rearranged IgH gene). Indeed, we were able to amplify the p53 gene fragment from the water boiled extracts in all of the normal sized tissues but in only 66% of the biopsy specimen sized samples. Furthermore, in half of the latter successful cases fainter bands were observed. The DNA from the presumed small population of monoclonal B cells also failed to be amplified in 17% of the samples, and yielded fainter bands in 80% of the positive cases, examined on normal sized B cell non-Hodgkin's lymphoma tissues. However, all of the biopsy specimen sized tissues were amplified successfully, probably due to the more sensitive semi-nested PCR, although it is also possible that the clonal population was more numerous in these cases. When 1/2, 1/4, 1/8 and 1/16 subdivisions of the normal sized lymphoma tissue were processed, the samples prepared by the water boiling method produced clearly weaker bands on the 1/8 and 1/16 portions (data not shown). In contrast, we did not experience any difference when amplifying HPV 16 on normal or biopsy specimen sized cervical samples, where the target number seems to be above the limit that can cause discernible differences (cervical cancer cells may contain one to 50 copies of HPV per cell).

Preparing the tissue samples by boiling in Chelex-100 solution produced sufficient amount of DNA to be amplified at the same intensity as from those samples prepared by proteinase K based methods, regardless of the tissue type, size, or target sequences used. Chelex-100 is a chelating resin that preferentially chelates polyvalent metal ions. Sample processing by Chelex has been successfully employed for a small number of cells,[65] for forensic type materials,[66] and for paraffin wax embedded tissues.[4, 5, 23] The effect of Chelex, which prevents the degradation

of DNA, is thought to be due to chelating metal ions, which may act as catalysts in the breakdown of DNA at high temperatures in low ionic strength solutions.[65, 66] Indeed, templates prepared by Chelex boiling in the present study were shown to be better preserved by electrophoretic examination than those prepared by the water boiling method (Figure 3, lanes A and B). It is also possible that Chelex may bind other substances, e.g. blood derived products, which could inhibit the PCR itself.[66]

In the present study, a 3 hour proteinase K digestion was also capable of yielding a sufficient amount of template DNA to amplify any of the targets in all of the cases. Although increasing the time of the digestion substantially improves the yield of the DNA,[2] for routine purposes this does not seem to be essential. However, to avoid phenol-chloroform extraction and to use supernatant fluid directly, it is advisable to apply non-ionic detergents in the digestion buffer instead of sodium dodecyl sulphate, which was reported to inhibit *Taq* polymerase.[20, 67] In this experiment, adding Chelex to proteinease K digested samples (Method D) proved to have no noticeable influence on the sample quality.

The results of the present study also indicate several limits regarding the size of the products that can be amplified from various extracts. Water boiled samples are extremely degraded and were found to allow amplifications up to only 400 bp fragments in the present study. With a better preservation of the target DNA, it was possible to amplify fragments up to about 650 bp from Chelex boiled material and up to 1000 bp from proteinase K digested material, although with compromised specificity. Nevertheless, this amplification product size is unlikely to be used for diagnostic purposes.

Dewaxing seems to have no effect on the successful release of DNA from paraffin wax embedded tissues. No difference was found when tissues were boiled with or without previous deparaffinisation, which is in agreement with other observations.[4, 21] Although tissues were dewaxed before proteinase K digestion, other investigators have eliminated this step without any effect on the yield or quality of DNA obtained.[2, 19]

All extracts were reamplified for their relatively short (80-166 bp) specific targets, after storing them at -20°C for three months. However, weakening in the product intensity was observed for the 408 bp fragment, and failure to reamplify the 647 bp fragment was noted from Chelex boiled samples. This decreased amplification ability was also reported in stored proteinase K digested samples, and is likely to be due to degradation of the template DNA.[20]

In conclusion, the simplest water boiling method may fail to produce a sufficient quantity and quality of template from formalin-fixed, paraffin-embedded tissues, particularly when the starting material is small, the target molecules are expected to be of low copy number, or larger fragments are to be amplified. DNA prepared by Chelex boiling is of a quality that can be amplified as successfully, in all practical aspects, as that extracted by proteinase K digestion. As the procedure is simpler, requires less time and less manipulation -thus decreasing the possibility of contamination- it would be helpful for the large scale, routine treatment of paraffin wax embedded samples for the use in PCR.

2. Screening for the presence of human papillomaviruses in archive lung carcinoma samples by polymerase chain reaction.

To test the ability of detecting the presence of possible viral DNA in archive fixed tissue samples we screened for human papillomaviruses in archive lung carcinoma samples by PCR. The potential role of HPV in the development of lung cancers emerged after histological changes, suggesting HPV infection, were described in the metaplastic bronchial mucosa adjacent to squamous cell carcinomas.[68-70] Early studies searching for HPV presence in lung cancers using in situ hybridization and Southern blotting reported identification of high risk and low risk type viruses, with a variable rate of positivity between 4.2% and 18.2%.[43-46]

In our work, we used the more sensitive PCR method with primers capable of detecting at least eight types of HPV most frequently found in HPV-associated mucosal tumours. The sensitivity of our method is about 0.1 viral copy/cell,[64] which is approximately the same as that of Southern blotting,[71, 72] and is higher than the 10-20 copies/cell detection limit of ISH.[46, 71]

We were able to show that DNA extracted from archive lung tissue samples are suitable for screening for viral sequences, as we proved that sufficient quality and quantity DNA was present in the reactions, because we were able to amplify the single-copy p53 human gene. Positive controls (cervical cancer and condyloma acuminatum specimens, which were processed in the same way as the study material) always showed positivity and negative controls gave negative results. However, we did not detect HPV in any of the squamous and large cell cancer samples. In the view of the above mentioned factors, technical error is unlikely and we strongly believe that our results were due to the absence of the examined HPV types. It is also possible that our samples harboured less than 0.1 viral copy/cell, which was the

maximum sensitivity of the PCR. However, a basal epithelial cell of infected cervical epithelium is known to carry 50-100 copies of the HPV genomes per cell, and if HPV has a causative role in bronchial carcinogenesis, it should be present above the PCR detection limit in the tumour.

With regard to HPV detection in lung cancers, based on our initial findings a number of studies were carried out on HPV detection in bronchopulmonary carcinomas, the principal data of which is summarised in Table 2.[73-92] The prevalence of HPV detection in these series of lung carcinomas varies from 0%[74, 83, 87] to 79%.[86] The high prevalence of HPV detection in lung carcinomas in countries such as Japan,[82, 86, 90] China,[79] Greece[85] and Finland[80, 81] may reflect the possibility of a geographic variability of HPV infection associated with these lesions. However, there is also a considerable variation between studies originating from the same study population, e.g. two studies on Greek patients reported 69%[85] and 0%[87] of HPV prevalence. The six studies reporting HPV prevalence in Japanese population clearly fall into two categories based on the origin of the study cohort. A surprisingly high HPV prevalence has been described in the subtropical island of Okinawa, South Japan, where three studies (from the same research group) reported a HPV prevalence of 36.2%,[82] 78.3%,[86] and 33.8%.[90] However, studies on populations from mainland Japan reported HPV occurrence of 10.3%[73] (Okayama), 8.3%[77] (Hokkaido) and 0% (our study, Kagawa). It is of note, that a study from Taiwan, close to Okinawa also found a 54.6% of HPV occurrence in non-smoking Taiwanese women (although the prevalence in non-cancer controls was also high, 26.7%).[89] Studies on larger series of patients usually report a low frequency of HPV detection [e.g. 5/185 (2.7%),[88] 2/108 (1.8%)[75] and 0/85 (0%),[74]], which is in agreement with our results.

There is also a clear heterogeneity regarding the source of the tissue (frozen or fixed) and the detection method used. Most studies used PCR with consensus primers amplifying HPV DNA in the L1 and/or E6 or E6/E7 or E1 regions. The primer systems used in the various studies are important considerations, because the genome of the high risk type viruses may suffer partial deletion during integration into the host genome. However, the E6 and E7 open reading frames, which are responsible for the transforming ability of HPV, are virtually always conserved and expressed in most cancer cells.[28, 29] Since our primers were located on the E6-E7 open reading frames, we should have detected the virus, had it been present. Some authors have used two sets of primers, nested PCR, or PCR with specific type primers to improve the

Author	Country/year	Tissue	PCR	Total lung carcinomas		Squamous cell carcinomas		Other carcinomas	
				Cases	HPV (%)	Cases	HPV (%)	Cases	HPV (%)
Ogura *et al*[73]	Japan/1993	frozen	PCR E6 + DB	29	3 (10.3)	NS		NS	
Szabó *et al*	**Japan/1994**	**fixed**	**PCR E6/E7**	**47**	**0**	**40**	**0**	**7**	**0**
Shamanin *et al*[74]	Germany/1994	frozen	PCR L1/E1 + SB	85	0	34	0	51	0
Fong *et al*[75]	Australia/1995	frozen	PCR L1 + PCR E6/E7	108	2 (1.8)	43	2 (4.6)	65	0
Al Ghamdi *et al*[76]	UK/1995	fixed	PCR E1 + PCR TS	40	3 (7.5)	21	2 (9.5)	19	1 (5.2)
Kinoshita *et al*[77]	Japan/1995	frozen	PCR E6 + SB	36	3 (8.3%)	10	1 (10)	26	2 (7.7)
Thomas *et al*[78]	France/1995	frozen	PCR E6/E7 + nPCR E6/E7	31	5 (16.1)	18	2 (11)	13	3 (23)
Li *et al*[79]	China/1995	frozen	PCR E6/E7 + DB	50	16 (32.0)	27	13 (48.1)	23	3 (13)
Nourva *et al*[80]	Finland/1995	fixed	PCR L1 + nPCR TS	22	8 (36.4)	0	0	22	8 (36.4)
Soini *et al*[81]	Finland/1996	fixed	PCR L1 + nPCR TS	43	13 (30.2)	28	8 (28.6)	15	5 (33.3)
Hirayasu *et al*[82]	Japan/1996	fixed	PCR E6/E7	94	34 (36.2)	43	34 (79.1)	51	0
Welt *et al*[83]	Germany/1997	fixed	PCR L1 + nPCR L1	38	0	32	0	6	0
Bohlmeyer *et al*[84]	USA/1998	fixed	PCR L1 + SB	34	2 (5.9)	34	2 (5.9)	0	0
Papadopoulo *et al*[85]	Greece/1998	frozen	PCR L1 + SB	52	36 (69.0)	52	36 (69.0)	0	0
Tsuhako *et al*[86]	Japan/1998	fixed	PCR E6/E7	23	18 (78.3)	0	0	23	18 (78.3)
Gorgoulis *et al*[87]	Greece/1999	fixed	nPCR L1 + TS + DB	68	0	31	0	37	0
Clavel *et al*[88]	France/2000	frozen	Hybride Capture II.	185	5 (2.7)	101	2 (2)	74	3 (4)
Cheng *et al*[89]	Taiwan/2001	fixed	nPCR L1	141	77 (54.6)	58	31 (53.4)	83	46 (55.4)
Miyagi *et al*[90]	Japan/2001	fixed	PCR E6/E7	121	41 (33.8)	59	29 (49.1)	62	12 (19.3)
Miasko *et al*[91]	Poland/2001	NS	PCR NS	40	4 (10)	22	1 (4.5)	18	3 (16.6)
Kaya *et al*[92]	Turkey/2001	fixed	PCR NS	26	3 (11.5)	26	3 (11.5)	0	0

Table 2. Chronological HPV identification in lung carcinomas by using different PCR systems. Present study is in **bold**. HPV: human papillomavirus, PCR: polymerase chain reaction, E6/E7/L1: E6/E7/L1 region of the HPV genome, TS: type specific primers, nPCR: nested PCR, SB: Southern blotting, DB: dot blot, NS: not specified.

rate of HPV detection with the possibility of screening novel HPV types associated with lung carcinoma. Moreover, Southern blot and dot blot after PCR also have been used. All of these methods have a very high sensitivity and specificity. A possible explanation for the high detection rate in some of the above studies may lie in this very high sensitivity: as million copies of the target HPV sequence is generated in each PCR run, PCR carry-over is a high concern in these experiments. None of the studies, reporting high prevalence of HPV presence, proved that the detected HPVs are policlonal (i.e. belonging to a different HPV strain), which would be a clear indicator for the reliability of the studies.

In summary, although we were able to show that archive lung samples are suitable sources of DNA, our lung cancer cases were found not to be associated with HPV presence and later studies on the same topic produced comparable results. The significance of HPV detection in lung tumours is still unclear, as it is still unknown whether HPV infection, if present, occurs before or after the tumour has developed. It is of note that some authors have reported immortalisation of human bronchial cells by HPV types 16 and 18, but these transfected cells have not given rise of tumours in the athymic mice.[93, 94] Thus, the presence of HPVs and their role, if any, in the tumour genesis remain to be elucidated in lung carcinomas.

3. Searching for disease causing mutations in archive samples of patients with hypertrophic cardiomyopathy.

To test the ability of detecting possible disease causing mutations in archive tissue samples we screened for mutations in archive tissue samples of patients with hypertrophic cardiomyopathy.

The first mutation we identified in case No1. is an Arg719Gln mutation in the beta myosin heavy chain gene, which is the first beta myosin heavy chain gene mutation identified in a Hungarian patient so far. Mutations in the beta myosin heavy chain gene are frequent cause of HCM, making up about 30-35% all of the cases. Since the first mutation reported in 1990[49] there were more than 50 mutations identified in the gene. Mutations affecting the beta myosin heavy chain gene are almost exclusively missense mutations, located in the first 23 exons of the gene, coding for the globular head and hinge region of the myosin protein.[60] The clinical manifestation of the mutations can be either malignant or benign. For instance, the Arg403Glu mutation of the beta myosin heavy chain gene was shown to be associated with high

penetrance, increased risk of sudden cardiac death and marked septum hypertrophy.[61, 95] A similar clinical phenotype has been observed with mutations Arg719Trp[96] and Arg453Cys.[61] In contrast to malignant beta myosin heavy chain gene mutations, mutations such as Leu908Val have been found to be associated with low penetrance, benign clinical course and low incidence of sudden cardiac death.[97]

The affected codon in our family, codon 719 of the beta myosin heavy chain, is part of a domain that has been proposed to interact with the myosin light chains in the sarcomere.[98] Arg719 is invariant in adult mammalian muscle myosin heavy chain sequences. Another mutation, Arg719Trp, has been described in the same position[96, 99] in several, non-related families, indicating a possible higher mutation rate of codon 719.

In the literature, there are a number of other reports describing HCM families with the same Arg719Gln mutation. In one family, which was of Hispanic origin, four mutation carriers were identified.[100] There was only one affected child in the family in whom the diameter of the septum was 16 mm, while septal thickness ranged 20-27 mm in adult mutation carriers. The disease was apparently benign in the family, as the affected family members had no symptoms; ECG, Holter recordings and exercise test were normal. An extended family history of 64 family members was negative for sudden cardiac death. The second family, which was of Chinese origin showed a malignant phenotype.[101] The proband was a female who was diagnosed having HCM at the age of 25 when she presented symptoms of chest pain, palpitations, and syncope. Echocardiography showed moderate septal hypertrophy (15 mm), without significant left ventricular outflow tract obstruction and dilated left atria. The patient also developed atrial fibrillation. Two of her family members (her mother and one of her sisters) had similar clinical manifestations and died suddenly at the age of 38. No autopsy material was available to provide a genetic diagnosis in these cases. In another two North-American families the Arg719Gln mutation was associated with also a malignant phenotype.[102]

In the family we studied, the proband's premature sudden death at young age, the early manifestation of the disease with marked cardiac alterations and the 100% penetrance of the Arg719Gln mutation also suggest a malignant phenotype. The other mutation at the same codon, Arg719Trp, has been almost invariably associated with such a malignant phenotype,[96] similar to the case we described. The discrepancy between the two phenotypes caused by the

same mutation has been observed with other beta myosin heavy chain mutations as well in patients with hypertrophic cardiomyopathy.[61, 103] A theoretical explanation could be that two mutations are present in an affected family, which alters the originally benign nature of a single mutation. However, compound heterozygosity for two mutations, although reported in HCM,[99] is quite rare. Another explanation may be the different genetic background that may affect phenotypic expression via modifier genes.

The second mutation we identified in case No 2. is a new mutation in the cardiac myosin binding protein C in our proband and her family. Myosin binding protein C is a member of the myosin binding proteins, which are present in all striated muscles.[104] The protein is located in the "crossbridge" (C) zone of the sarcomere as 7-9 bands, tangentially located to the horizontal axis of the sarcomere. The protein is consisted of 7 immunoglobulin-like and 3 fibronectine-like domains (C1-C10). In the cardiac specific isoform, an additional C0 domain is present at the N terminal end of the protein, and there is also an extra phosphorilable region between domains C1 and C2. Domains C8-C10 are responsible for binding to titin, while domain C10 is responsible for binding to myosin. According to experimental data, the protein has a dual role: on one hand it is responsible for the structural integrity of the protein (by binding to myosin and titin it provides a compact structure to the sarcomere); on the other hand it has a functional role by affecting the contractility of the sarcomere via phosphorilation. The myosin binding protein C gene, which is located on chromosome 11, is another important HCM gene, being responsible for about 30% of HCM cases. Since the first report on a myosin binding protein C mutation causing HCM in 1996,[53, 54] there has been about 30 other mutations described. Unlike other HCM genes, where missense mutations dominate, mutations affecting the myosin binding protein C gene are usually splice site mutations, leading to a truncated protein.[60] This truncation is almost invariably affects the distal C terminal domains of the protein where the essential myosin and titin binding domains are located.

The consequence of the Gln1226Stop mutation we identified is the loss of exons 34 and 35 and, in part, exon 33, which encode for the terminal 48 amino acids of the protein. As this region is part of domain C10, the titin and myosin binding property of the protein will be affected. This mutation is a novel *MYBPC3* mutation and it is also the first *MYBPC3* mutation identified in a Hungarian HCM patient so far.

Reports on patients harbouring a myosin binding protein C gene mutation usually indicate an age dependent penetrance and a benign phenotype. In a report on a cohort of myosin binding protein C gene mutation carriers it was shown that only 58% of adults under the age of 50 years, who had a mutation in the cardiac myosin binding protein C gene, had cardiac hypertrophy, and the disease was not evident before the age of 40 in about 45% of HCM patients.[105] This was in sharp contrast with beta myosin or troponin T gene carriers, where penetrance was close to 100% below 40 years of age. Although morphological differences were not clearly evident in myosin binding protein C gene patients, life expectancy was better as compared to troponin T or beta myosin heavy chain mutations. In a comparative report, prognosis was significantly better in myosin binding protein C mutant patients as compared with beta myosin heavy chain mutant patients as no death occurred before the age of 40, the age of onset of the disease was significantly delayed (41±19 vs. 35±17 years) and before 30 years of age, the phenotype was particularly mild because penetrance was low (41% vs. 62%), and maximal wall thickness was significantly lower (12±4 vs. 16±7mm).[106]

In our case the mutation was inherited by two of the proband's offspring, who are 34 and 18 years old at present. Both children are free of signs or symptoms of the disease, and have a normal ECG and echocardiography. The only alteration we noted is a mitral valve prolapse which was also present in non-carrier family members. These two children can be regarded as non-penetrant carriers for the moment, and should be followed up.

In summary we were able to demonstrate that fixed archive material is suitable for mutation detection. We detected two different point mutations in patients with hypertrophic cardiomyopathy. Both mutations are the first disease-causing mutations identified in Hungarian HCM patients so far.

SUMMARY AND CONCLUSIONS

As formalin-fixed paraffin-embedded tissue samples, stored in world-wide pathology archives, form an invaluable source for investigating human diseases, we aimed to examine the use of these routinely fixed archive samples in molecular genetic diagnostics. To investigate different aspects of the problem, we applied a two step approach. First, we compared four rapid DNA extraction protocols from formalin-fixed paraffin-embedded tissues. The ability of these methods to provide DNA suitable for PCR was investigated with various sets of primers, amplifying a single copy human gene, a viral sequence and a rearranged immunoglobulin gene. Several technical aspects of PCR were also examined, taking into consideration the size of the processed tissue, previous dewaxing, target sequence length and the reuse of the extracts.

Second, we tested DNA templates extracted from formalin-fixed, paraffin-embedded tissue in molecular genetic diagnostic procedures. We designed different projects for the above purpose, aiming that the various studies would be coherent scientific projects in themselves and would provide valuable scientific information beyond methodological issues. We have chosen the two most widely used molecular biological diagnostic procedures for the above goal, namely virus identification and mutation detection. For the former, we screened archive lung carcinoma samples for the presence of human papillomaviruses. For the latter, we searched for disease causing mutations in archive samples of patients with hypertrophic cardiomyopathy.

As new findings, we found that:

1. Chelex boiling, proteinase K digestion, and proteinase K digestion followed by Chelex boiling produce suitable templates for PCR from a large variety of paraffin wax embedded tissues. The simplest water boiling method may fail to produce a sufficient quantity and quality of template, particularly when the starting material is small, the target molecules are expected to be of low copy number, or larger fragments are to be amplified.
2. Although we were able to show that archive lung samples are suitable sources of DNA, the lung cancer cases we examined were found not to be associated with human papillomavirus (HPV 6, 11 16, 18, 31, 33, 52b, and 58) presence. We concluded that this finding did not support the potential role of these viruses in the development of lung cancers.
3. We demonstrated that archive tissue material is suitable for mutation detection as we identified two different point mutations (Arg719Gln mutation in the beta myosin heavy chain gene; and Gln1226Stop mutation in the myosin binding protein C gene) in archive samples of

patients with hypertrophic cardiomyopathy. The identified Gln1226Stop mutation in the *MYBPC3* gene is a novel hypertrophic cardiomyopathy mutation and both mutations are the first sarcomeric gene mutations identified in Hungarian patients with hypertrophic cardiomyopathy.

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APPENDIX 1. Oligonucleotide primers used in the study

Primer	Sequence	Target region	Size of PCR product (in bp)	Source
E81	CCTATCCTGAGTAGTGGTAATCTAC	P53 gene, exon 8	166	Miller *et al*[107]
E82	GTCCTGCTTGCTTACCTCGCTTAGT			
E71	CTCCTAGGTTGGCTCTGACTGT	P53 gene, exon 7	131	
E72	CAAGTGGCTCCTGACCTGGA			
E61	CACTGATTGCTCTTAGGTCTGGC	P53 gene, exon 6	144	
E62	AGTTGCAAACCAGACCTCAGGCG			
E51	TTCCTCTTCCTGCAGTAC	P53 gene, exon 5	214	
E52	GCCCCAGCTGCTCACCATCG			
E41	AAAACCTACCAGGGCAGCTAC	P53 gene, exon 4	99	
E42	CTCAGGGCAACTGACCGTG			
H1	ATTAGTGAGTATAGACATTA	HPV 16, ORF E6	110	Shibata *et al*[3]
H2	GGCTTTTGACAGTTAATACA			
Fr3A	ACACGGC(C/T)(G/C)TGTATTACTGT	IgH gene, V region	80-120	Wan *et al*[6]
LJH	TGAGGAGACGGTGACC	IgH gene, J region		
VLJH	GTGACCAGGGT(A/G/C/T)CCTTGGCCCCAG			
pU-1M	TGTCAAAAACCGTTGTGTCC	HPV ORF E6	231-268	Fujinaga *et al*[64]
pU-31B	TGCTAATTCGGTGCTACCTG	HPV ORF E6	228	
pU-2R	GAGCTGTCGCTTAATTGCTC	HPV ORF E7		
MYH7x19F	TCCTCCTACTTCCTTCTTGCC	Beta myosin heavy chain gene, exon 19	210	Watkins *et al*[61]
MYH7x19R	TTCTGGTGCACCCTCATACC			
MYBPC3x33F	GATGTGTCTCCCTGGGTCCCTG	Myosin binding protein C gene, exon 33	345	Carrier *et al*[108]
MYBPCx33R	GAGGACAACGGAGCAAAGCCC			

Abbreviations: HPV: Human papillomavirus; ORF: open reading frame; IgH: immunoglobulin heavy chain gene; V region: variable region; J region: joining region

APPENDIX 2. Citation Index

Sepp R, Szabó I, Uda H, Sakamoto H. Rapid techniques for DNA extraction from routinely processed archive tissue for use in PCR. *J Clin Pathol* 1994; 47 (4): 318-323.

Number of citations: 36 (as of September, 2003)

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APPENDIX 3. Photocopies of publications