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The Application of PCR and STR DNA Profiling for the Identification of Haematoxylin Eosin Histological Slides in a Case of Sample Mix-Up Involving Synonymous Patients

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Abstract

A good laboratory practice ensures that biopsy material is correctly identified and associated with a given patient. Nevertheless, there are cases where the proof of origin of a tissue sample may be questioned. In this case study we have identified the source of cervical cancer glass slide sections stained with H/E, (hematoxylin eosin), after the request of a patient of Northern Greek origin who suspected sample mix-up when she coincidentally found out that a synonymous patient was examined for cervical cancer at the same time period in the same hospital in Greece. The patient was prepared to legally challenge the administrators of the downstream chemotherapeutic regimen. A combination of organic gradient clean up and silica membrane method was used for DNA isolation. Powerplex-16® system (Promega U.S.A) was used to generate complete DNA profiles from histological slides and the reference blood sample collected from the patient. Histochemical slides often yield inadequate STR profiles for successful DNA typing. Complete profiling in this case could be attributed to the adequate removal of stain and fixatives inhibitors and the isolation of good quality DNA for PCR or STR, protocols. Matching of histochemical slide DNA with patient blood DNA prevented legal action.

Keywords

Powerplex-16®; DNA Profiling; Sample Mix-Up; Synonymy Stained; Histological Glass Slides

Introduction

Sample mix-ups are usually avoided in laboratories where good laboratory practice is applied. Nevertheless, the issue of correct association of the specimen with a patient has been addressed in some laboratory settings especially in hospitals or national reference centers where the work load is heavy [1, 8].

In the past, proof of origin of histological slides included the comparison of tissue markers of gender or ABO blood groups [23]. With the progress in molecular techniques the use of DNA-based polymerase chain reaction (PCR) techniques were based initially on the characterization of HLA polymorphisms [4, 19, 26] and lately Short Tandem Repeat (STR) markers have been proposed [20]. Although the application of multiplex-STR systems have been system of choice, complete

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identification profiles of sufficient discriminatory power was not always achieved [11]. Poor condition of DNA, trace inhibitors present and the existence of cancerous tissues have been held responsible for amplification artifacts such as preferential amplification, loss of heterozygosity and microsattelite instability present in histochemical slide DNA profiles [15, 21]. In order to overcome some of these complications, DNA extraction protocols based on Chelex micro concentrator or spin columns with variable amplification success have been suggested [2, 5, 15, 17].

In the following work we have applied an alternative protocol where an organic gradient is used conjunction with a silica membrane spin column method is used to isolate DNA from two eosin hematoxylin histopathological slides from a patient with cervical cancer. Powerplex 16® (Promega USA)-a highly discriminatory multiplex STR system- was employed for the achievement for matching profiles from the stained slides and a patient blood sample.

Case Study

A patient with origin from Northern Greece who was being treated at a Greek hospital for cervical cancer coincidentally found out that another patient with the same first and last name was treated at the same time period in the same hospital with a similar disease. The patient requested the investigation of association with the diagnostic histological and histochemistry samples as she was preparing to legally challenge the administrators of chemotherapeutic regime.

Blood and Histological Samples

In the following study the samples used for the DNA isolation and amplification were two fixed paraffin (approximately 2 years old) embedded H/E histochemical slides where the cervical cancer was detected that were prepared as described in relevant literature [6], 5 ml peripheral blood in EDTA from the patient of Northern Greek origin and from a reference sample.

DNA Extraction Methods

All the following steps were carried out in a laminar flow cabinet located in a pre-PCR laboratory separated from the post-PCR laboratory and from another pre-PCR laboratory where the reference sample extractions and amplifications were setup. Essential steps for DNA decontamination of the surfaces and the cabinets such as UV irradiation and bleach treatment were taken. Before cover slip removal the slides were decontaminated according to the method by Alonso et al. [2].

Each fixed paraffin embedded histological slide was placed in a volume of xylene that covered the entire slide. After 24 hours the slides were removed and the tissue was scraped into eppendorfs containing 1.0 ml xylene. Tissue samples remained in the solvent for 24 hours while they were periodically vortexed. At this stage the tubes were spanned down, the supernatant was removed and 1 ml octanol was added and left for another 24 hours while they were periodically vortexed. Next, the tubes were spanned down, supernatant was discarded and the pellet was washed once with absolute ethanol and once with isopropanol. At that stage the pellets were left to air dry for one hour. DNA isolation from the pellets was performed with NucleoSpin Tissue kit (Macherey-Nagel) according to the manufacturer's manual. DNA from blood samples was isolated with Nucleospin Blood Kit (Macherey-Nagel) according to manufacturer's instructions. To ensure even the slightest possibility of cross contamination tissue slide isolation and analysis preceded blood DNA isolation.

DNA Quantification with Q-PCR

DNA extracted was quantified using the Quantifiler Human DNA Quantification kit with the use of 7500 Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions.

DNA Amplification and STR Characterization

DNA isolated with the previous techniques was amplified with the use of AmpliTaq Gold® DNA polymerase and Powerplex-16® system (Promega U.S.A) on a Perkin-Elmer model 480 thermal cycler according to manufacturers' instructions. The analysis was performed on the ABI Prism 7000 Sequence detection system (Applied Biosystems) and the ABI Prism 7000 SDS Software v. 1.1. DNA used for the above amplifications varied between 0.5-1 ng.

Results and Discussion

DNA analysis from histological tissue slides is complicated by many factors. Primarily, the amount of DNA is expected to be low due to small amount of starting tissue material. Therefore, it was essential to apply for this DNA isolation protocol all necessary precautions taken to minimize background contamination. Secondly, the structure of DNA from histochemical slides is compromised due to fixation and other treatments

Table 1: Genetic Profiles and Loci Frequencies of the Histological Slides, Slides Analysed

LOCUS	Reference Alleles	Slide1 Alleles	Slide 2 Alleles	Patient Alleles	q	p	q^2	2pq	EGF
AMEL	X	X	X	X	-	-	-	-	-
	X	X	X	X	-	-	-	-	-
D5S818	9	9	9	9	0.0582				
	11	11	11	11		0.3003		0.0349	0.0349
D7S820	8	8	8	8	0.1714				
	12	12	12	12		0.1635		0.056	0.056
TH01	8	6	6	6	0.2516				
	9.3	7	7	7		0.1399		0.0704	0.0704
TPOX	8	8	8	8	0.5472				
	8	8	8	8			0.2994		0.2994
vWA	17	16	16	16	0.2123				
	18	17	17	17		0.2752		0.1168	0.1168
CSF1PO	10	12	12	12	0.3129				
	12	13	13	13		0.0503		0.0314	0.0314
D16S539	11	9	9	9	0.1305				
	12	9	9	9			0.017		0.017
D13S317	11	11	11	11	0.316				
	12	12	12	12		0.305		0.197	0.197
D8S1179	13	13	13	13	0.3491				
	13	14	14	14		0.2201		0.1534	0.1534
D3S1358	14	17	17	17	0.228				
	15	17	17	17				0.052	0.052
FGA	23	20	20	20	0.1226				
	24	24	24	24		0.1572		0.0385	0.0385
PENTA D	12	12	12	12	0.1447				
	12	13	13	13		0.1777		0.0514	0.0514
PENTA E	12	5	5	5	0.0409				
	13	17	17	17		0.0362		0.0148	0.0148
D21S11	30	28	28	28	0.1352				
	30	29	29	29		0.2657		0.072	0.072
D18S51	15	16	16	16	0.1336				
	19	17	17	17		0.0943		0.0251	0.0251
								PRCF	$2.13X10^{-19}$

Table 1: Summary of results listing the alleles and respective frequencies of the samples analysed that demonstates match between the patient and the slides. The loci listed are AMEL, D5S818, D7S820, TH01, TPOX, vWA, CSF1PO, D16S539, D13S317, D8S1179, D3S1358, FGA, PENTA D, PENTA E, D21S11, D18S51 as included in the Powerplex 16 kit (Promega). In column Reference Alleles the genotype of the patient sample is listed. In columns Slide Allele 1 and Slide Allele 2 the genotypes of the slide samples are listed. Frequencies of are listed in q, p, q^2 and 2pq columns as described by Hardy-Weinberg equilibrium. EGF is the expected genotype frequency and PRCF is the product rule combined frequency for the profile of both slides and patient sample. The allele frequencies presented a in this study are presented as included in literature [16].

during slide preparation [2, 10, 14]. In previous studies on unstained slides or paraffin shavings, DNA damage due to fixation compromised DNA amplification efficiency, but it did not completely prevent STR amplification [11, 24]. One the other hand, some reports point out that even trace amounts of histochemical dyes might interfere with proteinase K digestion [9, 18] and/or down stream inhibit PCR [3, 12]. Furthermore DNA preservation from histological preparations is thought to be influenced by the type of stain and staining protocols employed [5].

Successful DNA amplification from eosin-hematoxylin histological slides has been difficult to achieve in the past. Burton et al. [7] suggested that hematoxylin-eosin should be avoided if DNA analysis is applied downstream. In contrast, Banaschak et al. [5] showed that successful removal of hematoxylin eosin during a Chelex based DNA extraction permits the amplification of a commercial triplex kit (AmpFLSTR Blue, ABI, Weiterstad, Germany). Chelex is a widely used resin in forensic genetics nevertheless its usage is not always straightforward permitting inhibitory effects when the supernatant is not carefully aspirated. Alternatively, a combination of phenol-chloroform/Centricon method was employed [5] where mitochondrial DNA segments were successfully amplified but failed in multiplex-STR amplification. Finally, a spin column protocol has been successfully employed in a related paternity report involving histological slides [17]. Nevertheless, in our case, we included as a precaution an initial step for the removal of bulk inhibitors in the form of a gradient of non-polar to polar solvent instead of phenol-chloroform for the clean up of tissue. Dye removal was verified by our observations that the tissue pellet after treatment with the gradient of organic solvents appeared to be clear of any coloration. Silica spin columns have been useful for sample isolation and inhibitor removal from a great range of forensic specimens including paraffin embedded tissues, therefore they were considered as an ideal choice for the second step of our isolation. In addition the use of silica membrane formats allows the possibility of automation which is a great advantage for laboratory environments with heavy work load [7].

Despite the complications involved in DNA isolation from histochemical slides, the protocol presented here produced DNA of adequate quality (0.3-0.5 ng/μl as detected by Q-PCR) for the amplification of complete Powerplex 16® STR profiles. These led to a match from two histochemical slides and a blood sample from the patient with PRCF= $2.13X10^{-19}$ (Table 1). The result discouraged the patient to proceed to legal action.

Although microsatellite alterations are well documented in many cancer tissues analysed with STR systems it is sometimes not clear whether these alterations are due to cellular events or result from the influence of sample condition. In the present analysis we observed no apparent microsatellite alteration. This suggests that no microsatellite instability or loss of heterozygosity occurred in the tissues examined. This also supports that the absence or low concentration of inhibitors had no effect on the downstream PCR. Nevertheless, additional experiments involving several types of tissue samples and different types of dyes might reveal the threshold of PCR inhibition and the universality of the method above.

High quality DNA profiles were imperative in this case because the issue of synonymy was involved. In Greece, naming sometimes follows local customs associated with family relatedness. Therefore one cannot exclude the fact that synonymous individuals may exhibit a degree of genetic proximity and this way influence the decision on identification if partial profiles were obtained [28]. Interestingly, in a previous study concerning a U.S database two unrelated individuals shared 9 out of 13 STR autosomal markers [27]. It has been argued by certain authors that to resolve such issues profiles should exceed 20 autosomal STRs [27, 28], however population data for Greek populations exceeding 16 autosomal STR markers is not currently available [16, 22, 25]. Nevertheless, successful resolution of 15 STR's provided exceeds dramatically the partial matching observed in the previous database report.

The above work produced a low cost, reliable, high resolution and potentially automated protocol for the analysis and provenance of eosin hematoxylin histochemical slides. Furthermore, it demonstrates the value of high discriminatory profiles in resolving the origin of synonymous samples in a clinical setting.

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