Int J Legal Med (2011) 125:327–332
DOI 10.1007/s00414-010-0443-7

ORIGINAL ARTICLE

Tumoural specimens for forensic purposes: comparison of genetic alterations in frozen and formalin-fixed paraffin-embedded tissues

Viviana Ananian · Pamela Tozzo · Elena Ponzano · Donato Nitti · Daniele Rodriguez · Luciana Caenazzo

Received: 14 December 2009 / Accepted: 4 March 2010 / Published online: 6 April 2010

Abstract In certain circumstances, tumour tissue specimens are the only DNA resource available for forensic DNA analysis. However, cancer tissues can show microsatellite instability and loss of heterozygosity which, if concerning the short tandem repeats (STRs) used in the forensic field, can cause misinterpretation of the results. Moreover, though formalin-fixed paraffin-embedded tissues (FFPET) represent a large resource for these analyses, the quality of the DNA obtained from this kind of specimen can be an important limit. In this study, we evaluated the use of tumoural tissue as biological material for the determination of genetic profiles in the forensic field, highlighting which STR polymorphisms are more susceptible to tumour genetic alterations and which of the analysed tumours show a higher genetic variability. The analyses were conducted on samples of the same tissues conserved in different storage conditions, to compare genetic profiles obtained by frozen tissues and formalin-fixed paraffin-embedded tissues. The importance of this study is due to the large number of specimens analysed (122), the large number of polymorphisms analysed for each specimen (39), and the possibility to compare, many years after storage, the same tissue frozen and formalin-fixed paraffin-embedded. In the comparison between the genetic profiles of frozen tumour tissues and FFPET, the same genetic alterations have been reported in both kinds of specimens. However, FFPET showed new alterations. We conclude that the use of FFPET requires greater attention than frozen tissues in the results interpretation and great care in both pre-extraction and extraction processes.

Keywords Forensic genetics · Microsatellite instability · Loss of heterozygosity · Tumoural specimens · Paraffin-embedded tissues · Frozen tissues

Introduction

Tumour tissue specimens obtained for diagnostic purposes represent a large source of biological material available for genomic studies, and in certain circumstances, such as identification of unknown corpses in mass disasters [1] or DNA paternity testing in case the putative father is not available, they are the ultimate DNA resource available for genetic analysis. It is known that carcinogenesis is a multistep process in which cells accumulate genetic alterations as they progress to a more malignant phenotype.

Genetic instability caused by mutations of proto-oncogenes, tumour suppressor genes, apoptosis and mismatch repair (MMR) genes correlates with genome-wide accumulation of

Electronic supplementary material The online version of this article (doi:10.1007/s00414-010-0443-7) contains supplementary material, which is available to authorized users.

V. Ananian · P. Tozzo (✉) · E. Ponzano · D. Rodriguez · L. Caenazzo
Department of Environmental Medicine and Public Health, Legal Medicine Unit, University of Padova,
Via Falloppio 50,
35121 Padua, Italy
e-mail: pamela.tozzo@unipd.it

D. Nitti
Department of Oncological and Surgical Sciences,
University of Padova,
Via Gattamelata 64,
35121 Padua, Italy

genetic mutations which can also involve the short tandem repeat (STR) loci used as markers in forensic practice [2]. Therefore, in the determination of genetic profiles, it is important to evaluate the effects of genetic alterations of STR loci, such as loss of heterozygosity (LOH) and microsatellite instability (MSI), in order to rationalise the use of cancer tissues for forensic purposes and, when necessary, select the polymorphisms and the type of samples to be used for the analysis [3].

The MSI phenomenon is characterised by allelic insertion: according to the NCI recommendations, extra alleles at ≥5/15 loci designate a tumour as high-frequency MSI, whereas tumours with extra alleles at <5/15 loci are designated as low-frequency MSI (MSI-L). This phenomenon was initially observed by three research groups [4–7] in patients with the hereditary non-polyposis colon cancer and was attributed to the loss of MMR function by somatic inactivation of the wild-type allele (MLH1, MSH2, MSH6) [8–10].

The LOH phenomenon is characterised by allelic deletion, with partial or complete loss of one allele. It is important to differentiate partial loss of heterozygosity (pLOH), in which the intensity of the tumour allele is >50% decreased but still detected by the electropherogram, from complete loss of heterozygosity (cLOH), which results in a heterozygote locus being incorrectly classified as homozygote. Only tumours showing cLOH events, as well as MSI, can be incorrectly genotyped, complicating the forensic evaluation [3].

Formalin-fixed paraffin-embedded tissues (FFPET) represent a great source of archival biological material available for genetic studies in the forensic field and, in several cases, the only DNA resource available [11, 12].

The quality of DNA extracted from FFPET often complicates the analysis or limits its efficiency. Many studies have demonstrated that formaldehyde (H_2CO), the main constituent of formalin, is responsible for DNA denaturation and cross-linking reactions. Furthermore, prolonged fixation periods lead to DNA fragmentation, especially when specimens are stored in unbuffered formalin. Therefore, the degradation of the DNA extracted from these samples and the alterations caused by formalin–DNA interaction lead to partial or total failure of the polymerase chain reaction (PCR) amplification process, resulting in partial or absent STR profiles. Moreover, pre-extraction procedures to remove paraffin from fixed tissues seem to be an important step in order to optimise the efficiency of the PCR reaction [13–19].

In this study, we evaluated the use of tumoural tissue in forensic genetics. The importance of this study is represented by the great number of polymorphisms analysed for each sample (39 polymorphisms for each of the 122 samples), as well as the comparison of genetic profiles obtained from frozen tissues and FFPET.

Materials and methods

Samples

Our study was performed on 122 tissue samples obtained from 34 individuals and collected from 1994 to 2002, divided as follows:

- 36 samples from 12 individuals (all female) with breast cancer. Of these samples, 12 were healthy tissue, 12 were frozen tumoural tissue and 12 were FFPET tumoural tissue
- 36 samples from 12 individuals (five females and seven males) with gastric cancer. Of these samples, 12 were healthy tissue, 12 were frozen tumoural tissue and 12 were FFPET tumoural tissue
- 50 samples from ten individuals (four females and six males) with colorectal cancer. Of these samples, ten were healthy tissue, ten were frozen tumoural tissue, ten were FFPET tumoural tissue, ten were frozen liver metastasis tissue and ten were FFPET liver metastasis tissue.

Healthy and pathological frozen samples were stored at −80°C. The FFPET samples were fixed in 10% formalin, processed routinely through dehydration in graded ethanol, cleared in xylol and embedded in paraffin blocks.

Pre-extraction treatment of fixed tissues: deparaffinisation method

For each paraffin block, five 10-μm-thick sections were cut and collected in a 1.5-ml microtube. One millilitre xylene was added to each tube and kept at room temperature overnight to remove the paraffin completely. The tubes were then centrifuged at 15,000 rpm for 2 min, and the supernatant was discarded.

The pellet was rehydrated with descendent graded ethanol washes (500 μl pure ethanol, 500 μl 90% ethanol, 500 μl 80% ethanol, down to 10% ethanol).

Each washing step consisted of 2 min of incubation at room temperature and centrifugation at 15,000 rpm for 1 min.

After the ethanol washes, the tissue pellet was suspended again in 1 ml distilled water for 30 min at room temperature.

DNA extraction

DNA extraction from both frozen and FFPET samples was performed with QIAamp® DNA Microkit following the procedures described by the manufacturer (QIAGEN).

About 30–50 μg of DNA was recovered in 30 μl of a final solution.

DNA amplification

DNA amplification for each sample was performed using five different amplification kits: AmpF/STR® Identifiler™, AmpF/STR® Profiler Plus™, AmpF/STR® Minifiler™, AmpF/STR® Yfiler™ PCR Amplification Kits (Applied Biosystem) and Mentype® Argus X-UL PCR Amplification Kit (Biotype), following the manufacturers' recommendations, and NC01 miniplex [20] for a total of 39 polymorphisms, as synthetically reported in Table 1.

PCR reaction was conducted using the GeneAmp® PCR System 9700.

The markers D8S1179, D21S11, D7S820, CSF1PO, D3S1358, D13S317, D16S539, D2S1338, VWA, D18S51, D5S818, FGA and Amelogenin are common to different kits.

The amplified alleles were separated by capillary electrophoresis using ABI PRISM 3130 GENETIC ANALYZER. As internal standard, Gene-Scan-500 LIZ® was used for Identifiler, Minifiler, YFiler Kits and NC01 miniplex, and Gene-Scan-500 ROX® was used for Profiler and Mentype Amplification Kits. The sizes of the PCR products were evaluated by the software GeneMapper 3.2 using allelic ladders as a comparison.

Results and discussion

Microsatellite instability was identified by comparing the genotype at each STR locus in the frozen tumour sample with the genotype obtained from the respective healthy frozen tissue.

Allelic deletion in the frozen tumour tissue as compared with its respective heterozygotic frozen healthy tissue, was classified as "LOH". Moreover, LOH was attributed to a peak intensity ratio (peak ratio in the tumour tissue/peak ratio in the healthy tissue) of <0.5.

Frozen samples

As summarised in Table 2, amongst the 44 tumour tissue samples analysed, 24 (54.6%) showed genetic alterations when compared with the genetic profile obtained from the DNA of the respective healthy tissue: five gastric cancer, four breast cancer and 6 colorectal cancer specimens and nine of the metastatic cancer specimens (colorectal liver metastasis).

The new alleles detected in addition to those displayed in the normal tissue were either contractions or expansions of one repeat unit, and all the samples have been designated as MSI-L.

The STR loci showing MSI were: D7S820, D3S1358, D13S317, VWA, FGA, D5S818 and DXS7132.

The main STR alteration in the analysed frozen tumour samples was LOH: D7S820, D19S433 and TPOX loci never showed LOH-type alterations, whilst D18S51 was the most frequently altered marker.

The fact that many markers are common to different kits can serve as a control to discriminate genetic alterations (when they are present in all the kits) from PCR artefacts.

Only three samples were classified as high-frequency LOH, showing LOH-type alterations at ≥33% of the heterozygote STR loci.

Amongst the 39 STR loci analysed, only TH01, D2S1338, TPOX and D19S433 never exhibited MSI- or LOH-type alterations, whilst the most altered loci were D18S51 (showing six pLOH and three cLOH events) and HPRTB (seven pLOH events).

The tumour tissue samples also revealed alterations in the determination of gender. Four of the 19 pathological male samples showed a strong imbalance between X and Y peaks of amelogenin with all kits used, so we classified them as an LOH at the amelogenin locus, maintaining the attribution of male gender, rather than an amplification failure because we used more than one kit and the samples amplified showed the complete genetic profile. In this study, different from Vauhkonen [21], the complete deletion

Table 1 STRs analysed for each sample in this study with the respective kits used

Kit	STRs
AmpF/STR® Identifiler™ PCR Amplification Kit (Applied Biosystem)	D8S1179, D21S11, D7S820, CSF1P0, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, VWA, TPOX, D18S51, D5S818, FGA, amelogenin
AmpF/STR® Minifiler™ PCR Amplification Kit (Applied Biosystem)	D13S317, D7S820, D2S1338, D21S11, D16S539, D18S51, CSF1PO, FGA, amelogenin
AmpF/STR® Profiler Plus™ PCR Amplification Kit (Applied Biosystem)	D3S1358, D5S818, D7S820, D8S1179, D13S317, D18S51, D21S11, FGA, VWA, amelogenin
AmpF/STR® Yfiler™ PCR Amplification Kit (Applied Biosystem)	DYS456, DYS389I, DYS390, DYS389II, DYS458, DYS19, DYS385a, DYS385b, DYS393, DYS391, DYS439, DYS635, DYS392, Y GATA H4, DYS437, DYS438, DYS448
Mentype® Argus X-UL PCR Amplification Kit (Biotype)	DXS8378, DXS7132, HPRTB, DXS7423, amelogenin
NC01 miniplex	D10S1248, D14S1434, D22S1045

of Y peaks of amelogenin was not found in the tumoural tissue samples.

The 17 Y-STR loci analysed were, on the contrary, extremely stable, showing no alterations in comparison to the healthy control tissues, as reported by Vauhkonen [21].

Amongst the 24 samples that presented altered genotypes, four gastric and eight colorectal samples showed MSI or complete LOH events, as summarised in Table 3: these specimens (27%) can therefore be incorrectly genotyped, complicating the forensic evaluation. In contrast to this, the condition of allelic imbalance has a relative importance for forensic purposes and is not likely to lead to incorrect genotyping.

Amongst the four gastric samples, two of them presented alteration in the profile in more than one locus.

Amongst the colorectal samples, one primitive colorectal tumour sample and three metastasis samples present more than one altered polymorphisms in their profile.

However, there are no alterations in the breast cancer samples.

So from a forensic genetic perspective, of all the frozen tumour samples, only six samples can lead to a wrong conclusion if they are used in the forensic casework.

To classify the results by alterations in each type of tissue, the tissue specimens which reported the greatest genetic instability in our study were the colorectal cancer tissue samples, primary and metastatic (liver metastasis), followed by gastric cancer tissue samples. The colorectal cancer tissue samples alone showed 62 of the 81 genetic alterations we detected, of which 42 were pLOH, 11 were cLOH and nine were MSI events. The gastric cancer tissue samples showed 11 alterations, of which three were pLOH, four were cLOH and four were MSI events. The breast cancer tissue samples, on the contrary, showed the most similar genotypes compared with the control tissues, showing only ten pLOH events, and were therefore the most reliable tumour tissue samples for forensic DNA profiling.

Furthermore, a comparison between DNA profiles of colorectal tumour tissue samples and their respective liver metastasis was performed, and three metastatic samples showed new alterations not exhibited by the primary tumour.

FFPET samples

In our study, we used five 10-μm tissue sections of each sample in order to obtain a sufficient amount of DNA; poor

Table 2 Comprehensive number of genetic alterations found in the tumour tissue samples analysed

Tissue	Gastric			Colorectal			Breast	Total
	pLOH	cLOH	MSI	pLOH	cLOH	MSI	pLOH	
D8S1179				2				2
D21S11		1		1				2
D7S820						2		2
CSF1PO				3	1			4
D3S1358				2				2
TH01								
D13S317		1	1			2		4
D16S539					1		1	2
D2S1338								
D19S433								
VWA		1	1	3		1		6
TPOX								
D18S51				6	3			9
AME	1			3				4
D5S818			1	1	1			3
FGA				1		2		3
DXS8378				2			2	4
HPRTB	1			4			2	7
DXS7423				4			2	6
DXS7132			1	3		2	1	7
D10S1248				2			2	4
D14S1434	1	1		2	3			7
D22S1045				3				3
Total	3	4	4	42	11	9	10	81

pLOH partial loss of heterozygosity, *cLOH* complete loss of heterozygosity, *MSI* microsatellite instability

Table 3 Comprehensive number of genetic alterations for each type of tumour (12 gastric tumour samples, ten primitive colorectal tumour samples plus ten metastasis samples, 12 breast cancer samples)

Tissue	Gastric	Colorectal	Breast	Total
Samples with MSI o cLOH	4/12	8/20	0/12	12/44

yield would be obtained from a poor cellular content, whilst overly thick tissue slices may contain a high quantity of PCR inhibitors.

Although the formalin fixation time is considered to be the main parameter responsible for DNA degradation in FFPET, the removal of the paraffin seems to be an important step to improve the quality of the DNA yield and the success of the amplification process.

Therefore, the samples were incubated overnight in xylol to completely remove the paraffin and then rehydrated with graded ethanol washes.

The extraction of DNA was revealed to be a critical step in FFPET. We followed Qiagen procedure using QIAamp® DNA Micro Kit [22]: this method is based on the use of columns in which the DNA binds selectively to the silica gel membrane, allowing us to obtain highly purified DNA.

Furthermore, we used high concentrations of proteinase k [23–25] and high incubation temperature for the digestion step [26].

The age of FFPET samples, collected from 1994 to 2002, does not seem to be related to the difficulty of extraction to obtain a good quality of DNA: in fact, we found that it was more difficult to obtain a good DNA profile from some samples collected from 1999 to 2001, such as the colorectal ones, whereas for gastric samples, we obtained good DNA profiles more easily from tissue collected between 1993 and 1994. In our opinion, this could be attributed to the treatment of the tissue samples before paraffin inclusion rather than the storage conditions or age of the sample.

In the DNA amplification, we added bovine serum albumin to the solution to contrast PCR inhibitors.

The best success rate of PCR amplification was obtained with gastric and breast cancer tissue samples, as we were able to define a complete DNA profile from each sample.

In the comparison between profiles obtained from frozen and FFPET samples, the same genetic alterations in terms of pLOH, cLOH and MSI were reported in both kinds of specimens, and both profiles of each sample were identical.

On the contrary, colorectal tissue samples required a great number of analyses in order to obtain DNA profiles.

From 50% of these samples, we obtained partial DNA profiles. The amplification process was repeated many times, adjusting the concentration of the template and the amplification parameters to optimise the PCR reaction. The difficulty of amplifying long fragments from FFPET is well known. In fact, our study confirmed low amplification efficiency for long-size alleles such as D7S820, CSF1PO, D18S51, FGA and D16S539. Only AMG locus (106–112 bp) was invariably amplified.

The amplification of these alleles was only possible using AmpF*l*STR Minifiler PCR Amplification kit, which uses primers closely flanking the STR repetitive regions of the DNA, allowing us to obtain smaller PCR products and therefore increasing the efficiency of the amplification process in which conventional STRs fail to amplify long alleles.

DNA profiles obtained from colorectal FFPET samples showed 19 new genetic alterations in addition to the ones detected in the respective frozen samples, of which 15 were pLOH, two were cLOH and two were MSI new events, as reported in Table 4.

Also, four samples were found to possess pLOH at the Y-chromosomal AMG locus, not reported in the frozen

Table 4 Genetic alterations reported only in FFPET samples related to the respective frozen samples (the polymorphisms that did not show alterations are not reported in the table)

Tissue	Gastric			Colorectal			Breast	Total
	pLOH	cLOH	MSI	pLOH	cLOH	MSI	pLOH	
D8S1179				2				2
D21S11				1				1
CSF1PO				2				2
D3S1358				1				1
D13S317				3				3
D16S539				1	2			3
D2S1338				2				2
VWA				2				2
TPOX						2		2
D18S51				1				1
Total				15	2	2		19

samples. Furthermore, one sample presented a cLOH at the Y-AMG locus, causing a false determination of gender.

Conclusions

Typing of autosomal STRs from malignant tissues in forensic settings warrants careful interpretation of the electropherogram, especially in regards to the most genetically unstable cancers (colorectal cancer). A wide panel of markers should be used in parallel to obtain reliable results from the STR analysis. An efficient removal of the paraffin from fixed tissues has increased the success of DNA extraction and amplification. The DNA extraction is a critical step for fixed tissues, and the extraction methods must be chosen carefully. Also, it is important to conduct the amplification process with a large number of multiple PCR kits, in order to obtain a genetic profile which is as complete as possible.

The application of mini-STR primers in fixed specimens, which are characterised by highly degraded DNA, has proven to be efficient in the amplification process in which conventional STRs fail to provide good profiles. In the comparison between the genetic profiles of frozen tumour tissues and formalin-fixed paraffin-embedded tissues, the same genetic alterations have been reported in both kinds of specimens. However, formalin-fixed paraffin-embedded tissues show new alterations: 15 pLOH, two cLOH and two MSI new events. Also, one specimen was found to possess a cLOH at the amelogenin locus, causing a false determination of gender. Therefore, the use of formalin-fixed paraffin-embedded tissues in forensic casework requires greater attention than frozen tissues, not only in the interpretation of results but also in the pre-extraction and extraction processes, supported by the selection of the most appropriate protocols for each situation.

References

1. Whitaker JP, Clayton TM, Urquhart AJ et al (1995) Short tandem repeat typing of bodies from a mass disaster: high success rate and characteristic amplification patterns in highly degraded samples. Biotechniques 18:670–677
2. Silva F, Gusmão L, Alves C et al (1997) Tetra- and pentanucleotide short tandem repeat instability in gastric cancer. Electrophoresis 18:1633–1636
3. Poetsch M, Petersmann A, Woenckhaus C et al (2004) Evaluation of allelic alterations in short tandem repeats in different kinds of solid tumors—possible pitfalls in forensic casework. Forensic Sci Int 145:1–6
4. Aaltonen LA, Peltomäki P, Leach FS et al (1993) Clues to the pathogenesis of familial colorectal cancer. Science 260:812–816
5. Thibodeau SN, Bren G, Schaid D (1993) Microsatellite instability in cancer of the proximal colon. Science 260:816–819
6. Ionov Y, Peinado MA, Malkhosyan S et al (1993) Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. Nature 363:558–561
7. Vauhkonen M, Vauhkonen H, Sajantila A et al (2005) Differences in genomic instability between intestinal- and diffuse-type gastric cancer. Gastric Cancer 8:238–244
8. Fishel R, Lescoe MK, Rao MR et al (1993) The human mutator gene homolog MSH2 and its association with hereditary non-polyposis colon cancer. Cell 75:1027–1038
9. Bronner CE, Baker SM, Morrison PT et al (1994) Mutation in the DNA mismatch repair gene homologue hMLH1 is associated with hereditary non-polyposis colon cancer. Nature 368:258–261
10. Vauhkonen H, Hedman M, Vauhkonen M et al (2004) Evaluation of gastrointestinal cancer tissues as a source of genetic information for forensic investigations by using STRs. Forensic Sci Int 139:159–167
11. Aviel-Ronen S, Qi Zhu C, Coe BP et al (2006) Large fragment Bst DNA polymerase for whole genome amplification of DNA from formalin-fixed paraffin-embedded tissues. BMC Genomics 7:312
12. Gilbert MTP, Sanchez JJ, Haselkorn T et al (2007) Multiplex PCR with minisequencing as an effective higher throughput SNP typing method for formalin fixed tissue. Electrophoresis 28:2361–2367
13. Greer CE, Peterson SK, Kiviat NB et al (1991) PCR amplification from paraffin-embedded tissues. Effects of fixative and fixation time. Am J Clin Path 95:117–124
14. Wang LT, Smith A, Iacopetta B, Wood DJ et al (1996) Nested PCR- SSCP assay for the detection of p53 mutations in paraffin wax embedded bone tumors: improvement of sensitivity and fidelity. J Clin Mol Pathol 49:M176–M178
15. Miething F, Hering S, Hanschke B et al (2006) Effect of fixation to the degradation of nuclear and mitochondrial DNA in different tissues. J Histochem Cytochem 54:371–374
16. Hewitt S, Lewis F, Cao Y et al (2008) Tissue handling and specimen preparation in surgical pathology. Arch Pathol Lab Med 132:1929–1935
17. Fracasso T, Heinrich M, Hohoff C et al (2009) Ultrasound-accelerated formalin fixation improves the preservation of nucleic acids extraction in histological sections. Int J Legal Med 123:521–525
18. Turrina S, Atzei R, Filippini G et al (2008) STR typing of archival Bouin's fluid-fixed paraffin-embedded tissue using new sensitive redesigned primers for three STR loci (CSF1P0, D8S1179 and D13S317). J Forensic Leg Med 15:27–31
19. Farrugia A, Keyser C, Ludes B (2009) Efficiency evaluation of a DNA extraction and purification protocol on archival formalin-fixed and paraffin-embedded tissue. Forensic Sci Int 194:e25–e28
20. Coble MD, Butler JM (2005) Characterization of new miniSTR Loci to aid analysis of degraded DNA. J Forensic Sci 50:43–53
21. Vauhkonen H, Hedman M, Vauhkonen M et al (2004) Typing of XY (male) genotype from malignant neoplastic tissue by the amelogenin-based sex test. J Forensic Sci 49:222–226
22. Legrand B, De Mazancourt P, Durigon M et al (2002) DNA genotyping of unbuffered formalin fixed paraffin embedded tissues. Forensic Sci Int 125:205–211
23. Romero R, Juston AC, Ballantyne J et al (1997) The applicability of formalin-fixed and formalin-fixed paraffin-embedded tissues in forensic DNA analysis. J Forensic Sci 42:708–714
24. Savioz A, Blouin JL, Guidi S et al (1997) A method for the extraction of genomic DNA from human brain tissue fixed and stored in formalin for many years. Acta Neuropathol 93:408–413
25. Evison MP, Smillie DM, Chamberlain AT (1997) Extraction of single-copy nuclear DNA from forensic specimens with a variety of post-mortem histories. J Forensic Sci 42:1032–1038
26. Forsthoefel KF, Papp AC, Snyder PJ et al (1992) Optimization of DNA extraction from formalin-fixed tissue and its clinical application in Duchenne muscular dystrophy. Am J Clin Pathol 98:98–104