Useful DNA typing using AmpFLSTR® Identifiler® Kit for formaldehyde-fixed paraffin-embedded (FFPE) tissues in early gastric cancer patient with lymph node metastasis

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Summary. After distal gastrectomy in a patient with early gastric cancer, 27 regional lymph nodes around the stomach were evaluated for the existence of metastasis. There was a OIIa+IIc type tumor 2.0x1.5cm in size in the gastric angle of the lesser curvature according to the Japanese Classification of Gastric Carcinoma (JCGC). Histologically, the lesion extended no deeper than the muscularis mucosae. The cancer stage was so early that no metastasis was expected to occur but a lymph node with metastasis was found in one lymph node along the common anterior hepatic artery (station No.8a). This histological type was a little different from that of a primary tumor. The doctor began to suspect that the lymph node with metastasis might have been from another patient by mistake. Therefore, DNA typing using the AmpFLSTR® Identifiler® kit was performed in formaldehyde-fixed paraffin-embedded (FFPE) tissues: 2 parts of gastric mucosa without cancer, one part of gastric mucosa with cancer, 4 lymph nodes without metastasis, and the lymph node station No.8a with metastasis. STR typing was successful in 6–14 STR loci and the detected STR type was the same in all samples. Compared with the STR type using DNA from the patient’s blood, the lymph node station No.8a was from the same patient. The lymph node with metastasis turned out to be not from another patient. Therefore, we suggest that DNA typing using the AmpFLSTR® Identifiler® Kit for FFPE samples is useful in such clinical cases.

Key words: DNA typing, AmpFLSTR® Identifiler® Kit, Gastric cancer, Lymph node metastasis, Formaldehyde-fixed paraffin-embedded (FFPE) tissues

Introduction

Recent advances in cancer screening, early detection and therapy are remarkable. Early-stage cancer and lymph node metastases that had been overlooked formerly are nowadays found. However, if early-stage cancer with metastasis is found, a possible mistake that someone may take a sample with metastasis for another patient’s should be avoided. In that case proof that the sample is not another patient’s is necessary.

In general, tissues from an autopsy and tumor tissue from the surgery room are stored in 10–20% formalin for a period of 3 days to 1 week, and then they are embedded in paraffin. Formalin is a solution of formaldehyde, and 10% formalin usually contains 3.5–3.8% formaldehyde (HCHO). Early formalin fixation preserves the cyt morphology in tissues very well, and the analysis of cell morphology using immunological techniques provides very important information. However, it is known that the preservation of cyt morphology by protein-protein and protein-nucleic acid crosslinks causes DNA fragmentation, degradation and crosslinks among nucleic acids (Impriam et al., 1987; Tokuda et al., 1990; Noguchi et al., 1997). Furthermore, the oxidation of formaldehyde by dissolved oxygen produces a strong acid, formic acid, so the fixation solution becomes very acidic, and then the DNA of formalin-fixed tissues is thought to be fragmented.

Generally, a polymerase chain reaction (PCR) that
amplifies the target region from fragmented and degraded DNA has been used in forensic DNA typing. Recently, personal identification using STR genotyping, including CODIS 13 core loci, has been used in various forensic laboratories in the world (Budowle et al., 1998, 2001; Butler, 2005) and STR genotyping has mainly been carried out with the commercially available AmpFSTR® Identifiler® PCR Amplification Kit (Applied Biosystems, Foster City, CA) (Applied Biosystems, 2001; Holt et al., 2002; Butler, 2005). Using this kit, the STR type in 15 STR loci, including the CODIS 13 loci and amelogenin gene, can be detected (Collins et al., 2004; Reid et al., 2004; Yoshida et al., 2005, 2006).

In this study, STR typing was performed using DNA samples from formaldehyde-fixed paraffin-embedded (FFPE) tissues containing a lymph node with metastasis in an early gastric cancer patient (Romero et al., 1997; Zehner and Lasczkowski, 2000). We report the successful personal identification by appropriate template DNA in PCR amplification with the AmpFSTR® Identifiler® Kit.

Case

The patient with early gastric cancer was female and 62 years old. She underwent a distal gastrectomy (Billroth-I) with D2 lymph node dissection according to the Japanese Classification of Gastric Carcinoma (JCGC) (Japanese Gastric Cancer Association, 1998; Koyanagi et al., 2004; Townsend et al., 2006). Macroscopic findings revealed no lymph node metastasis. The length of the resected lesser curvature and the duodenum was 11 cm and 0.5 cm, respectively (Fig. 1). There was a 0IIa+IIc type tumor 2.0x1.5cm in size in the gastric angle of the lesser curvature which could be seen with the naked eye. Histologically, there were well differentiated tubular adenocarcinomas, and moderately differentiated tubular adenocarcinomas with fused glands (Kumar et al., 2003). The latter tumor element formed nests and proliferated deeply as down growth, but the lesion extended no deeper than the muscularis mucosae (Fig. 2a,b). There were no venous invasions or lymphatic vessel invasions.

However, a lymph node with metastasis was found in the lymph node station No.8a out of 27 lymph nodes dissected. However, the histology of the lymph node with metastasis was a little different from that of the primary tumor (Fig. 3a,b). In the primary tumor, the tumor element with high-grade atypical cells showed CK7(+), CK20(-), MUC5(-), MUC6(-), CD10(+), CDX2(+), and p53(-), and the tumor element with low-grade atypical cells showed CK7(-), CK20(+), MUC5(-), MUC6(-), CD10(+), CDX2(+), and p53(-). However, the lymph node with metastasis showed CK7(+), CK20(-), MUC5(-), MUC6(-), CD10(-), CDX2(-), and p53(-). While both tumor elements of a primary tumor showed Ki-67(+) in almost all tumor cells, the percentage of Ki-67(+) cells was less than 50% in the lymph node with metastasis. In addition, while the gastric cancer of the primary tumor showed some degree of the intestinal type cell differentiation, the lymph node with metastasis did not show any apparent intestinal type character.

It is possible that this lymph node was metastasized from the cancer of another organ. However, these samples might be obtained from another patient. Therefore, we needed to examine whether these samples were obtained from the same patient or not. After giving informed consent from the patient, personal identification using the AmpFSTR® Identifiler® Kit was performed for FFPE tissues: 2 parts of normal gastric mucosa, one part of malignant gastric mucosa, 4 normal lymph nodes, and a lymph node with metastasis.

Materials and methods

Samples

Eight parts of FFPE tissues were used among the samples removed from the patient with early gastric cancer for histologic diagnosis: 2 parts of gastric mucosa without cancer, one part of tumor gastric mucosa with cancer, 4 lymph nodes without cancer, and the lymph node station No.8a with metastasis. These samples were fixed with 20% formalin neutral buffer solution (Wako, Osaka, Japan) for two weeks. The standard concentration of formalin is usually 10% and the usual period is 3 to 5 days. However, this fixed solution was not a simple formalin solution but a buffered formalin solution. Afterwards, these samples were embedded in paraffin and stored at room temperature. Furthermore, a peripheral blood sample was obtained from the same patient.

DNA extraction

After the surplus paraffin was removed from the
FFPE tissues blocks, about 30-40 mg from the blocks were cut using sterile disposal forceps and a scalpel, and placed in a 1.5-mL microcentrifuge tube. To remove the paraffin from the FFPE samples, 1 mL of 100% xylene was added to the tube, and mixed by vortexing for 15 sec. To melt the paraffin, the tissues were incubated at 1,200 rpm for 10 min at 50°C, centrifuged at 14,000 rpm for 10 min, and then the xylene was discarded. This xylene treatment was performed twice. Then, 1 mL of 99.5% ethanol was added to the pellet. The pellet was mixed by vortexing for 15 sec, and agitated at 1,200 rpm for 10 min at 20°C. It was centrifuged at 14,000 rpm for 10 min, and then the ethanol was discarded. These ethanol washings were performed twice. The pellet was left for about two hours so that the ethanol would evaporate. Then, two kinds DNA extraction methods were carried out about one sample. One is the RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Applied Biosystems) with slight modifications. The pellet was put into a solution 400 µL of digestion buffer, 20 µL of Proteinase K (QIAGEN, Hilden, Germany), and 20 µL of 1M dithiothreitol (DTT), and incubated for several hours at 1,200 rpm at 70°C. To achieve complete digestion, 20 µL of Proteinase K and 20 µL of 1M DTT were added to the pellet. The solution was incubated for 1–2 days at 1,200 rpm at 56°C. Then, DNA was extracted according to the manufacturer’s instruction. The other was the phenol/chloroform method (Sambrook et al., 1989). The pellet was put into a solution containing 300 µL of Buffer ATL (QIAGEN), 20 µL of Proteinase K (QIAGEN), and 10 µL of 1M DTT. The solution was incubated for 24 hr at 1,200 rpm at 56°C. Also, an extra 20 µL of Proteinase K and 20 µL of 1M DTT were added, and then the tubes were incubated for 1–2 days at 1,200 rpm at 56°C. Then, they were heated for 10 min at 99°C. DNA was extracted 3 times with phenol, followed by phenol-chloroform at a 1:1 ratio once, and precipitated by the addition of a 1/20 volume of 3M sodium acetate and a 2.5 volume of absolute ethanol. The pellet was washed with 70% ethanol and air-dried, and then purified by Microcon® YM-100 (Millipore, Bedford, MA).

Blood DNA was extracted from 100 µL whole blood using QIAamp® DNA Mini Kit (QIAGEN). The absorbance of the DNA at 260 nm and 280 nm was measured. Calculation of the DNA concentration is based on the absorbance at 260 nm (a solution with an OD$_{260}$ of 1 contains approximately 50 µg of DNA per milliliter). Furthermore, DNA purity is judged on the basis of the ratio of A$_{260}$ to A$_{280}$. A low ratio indicates contamination by protein.

**Analysis of STR loci**

To amplify D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818, FGA and the amelogenin loci, multiplex PCR using 1-800 ng of target DNA was performed with the AmpFSTR® Identifiler® PCR Amplification Kit, according to the manufacturer’s recommendations (Applied Biosystems, 2001). PCR amplification was carried out using a GeneAmp® PCR System 9700 Thermal Cycler (Applied Biosystems). The amplified products were electrophoresed on an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems). Analysis of the amplified products and allele designation was performed automatically using GeneMapper™ ID software (version 3.2, Applied Biosystems). To designate the detected alleles, the highest value was 6000 relative fluorescent units (RFU) and the lowest

![Fig. 2. Gastric cancer. A, x 20; B, x100](image)
value (threshold) was 150 RFU or 50 RFU.

Results

The DNA yields were 984 ng in the normal lymph nodes, 343 ng in normal gastric mucosa, 155 ng in the lymph node with metastasis, and 793 ng in the gastric cancer on average per 1mg of the FFPE tissues using the RecoverAll™ Total Nucleic Acid Isolation Kit. Using the phenol/chloroform method, the DNA yields were 5.7 µg in the normal lymph node, 3.5 µg in the normal gastric mucosa, 622 ng in the lymph node with metastasis, and 3.2 µg in the gastric cancer. Judging from the DNA quality and quantity, the phenol/chloroform method was better than the DNA extraction kit. The PCR amplification was performed using 1ng of DNA as a template according to the manufacturer’s protocol (Applied Biosystems, 2001), but no alleles were detected. Then, PCR was carried out with various concentrations of template DNA, so that the

Fig. 3. The lymph node station No.8a with metastasis. A, x 20; B, x 100

Fig. 4. A. Electropherogram of STR results obtained from gastric cancer using AmpF STR® Identifiler® PCR Amplification Kit. B. Electropherogram of STR results obtained from the lymph node station No.8a with metastasis.
alleles came to be detected. Concerning the concentrations of template DNA, the typing succeeded using 400 ng or 50 ng in the normal lymph nodes, 400 ng or 100 ng in the normal gastric mucosa, 300 ng in the gastric cancer (Fig. 4a), and 200 ng in the lymph node with metastasis (Fig. 4b). Furthermore, the number of STR loci with successful typing increased when the lowest peak amount was set from 150 RFU to 50 RFU (Fig. 5). STR typing using DNA from the gastric cancer was the most typical among all tissues. STR typing of 14 loci was successful with 150 RFU, but the typing of the D2S1338 locus was unsuccessful. With 50 RFU, STR typing of all loci was successful. In the lymph node station No.8a with metastasis, STR typing was successful in 6 loci with 150 RFU, and 9 loci with 50 RFU. The number of STR loci with successful typing was the lowest among all tissues.

STR typing by the detection of PCR products of short size was successful at 5 loci: D8S1179, D3S1358, D19S433, vWA, and D5S818 in all 8 tissues. However, STR typing did not succeed in several STR loci when detection of PCR product of long size was attempted. Our experience was that the size of the detectable PCR product in STR loci was short when the period of formalin fixation was long. When the formalin fixation was more than one week, the STR loci of more than 200 bp became undetectable. There are some reports in regard to such phenomena (Greer et al., 1991a,b; Yamada et al., 1994). A simple formalin dilution solution has an acidity of about pH 4, but buffered formalin is adjusted to pH 7.4-7.6 by a phosphate buffer. Neutral pH can prevent DNA fragmentation by formalin fixation. In this case, the tissue samples were stored in 20% buffered formalin for 2 weeks due to the “golden week”, a long holiday in Japan. It was higher than the standard concentration (10%) and longer than the usual period (3 to 5 days). However, it is thought that STR typing was successful because the fixed solution was not a simple formalin solution but a buffered formalin solution. Therefore, the tissue samples should be fixed in buffered formalin solution when considering DNA typing after histological diagnosis.

In this case, DNA was extracted from FFPE tissues using a DNA extraction kit and the phenol/chloroform method, and the absorbance of the DNA at 260 nm and 280 nm was measured. The ratio of A260 to A280 should be greater than 1.75. A lower ratio is an indication that significant amounts of protein remain in the preparation. The ratio by the phenol/chloroform method was higher than that by the DNA extraction kit. The phenol/chloroform method showed DNA with good quantity and quality, and PCR with good amplification, as compared with the DNA extraction kit. Moreover, the phenol/chloroform method used here involved a heat step after enzymatic digestion. Some studies showed that PCR-amplifiable DNA was yielded by using alkali plus heat pretreatment of the FFPE samples (Shi et al., 2002, 2004; Gilbert et al., 2007a). Crosslinking in this way can be heat-reversed. Therefore, it is thought that heat treatment is likely to uncross some of the DNA, making it easier to amplify. Accordingly, it is thought that the phenol/chloroform method with heat treatment is preferable to DNA extraction.

DNA amplification with the AmpF/STR® Identifiler® Kit requires 10 µL of DNA at a recommended concentration of 0.05-0.125 ng/µL. However, STR typing was not successful with this concentration at all, so we performed PCR amplification using various DNA concentrations, and STR typing turned out to be successful in many STR loci. As a result, too much or too little concentration of DNA was not appropriate for STR typing, and the appropriate concentration for PCR amplification differed among the tissues and the extracted DNA. The DNA concentration was measured by the absorbance of the DNA at 260 nm.

Discussion

Formalin fixation leads to 2 phenomena--crosslinking of protein-protein and protein-nucleic acid, and DNA fragmentation due to strand breakage. DNA fragmentation is caused because of the increased acidity of the solution for formalin fixation. Therefore, it is said that PCR amplification is difficult if the size of the PCR product is long (Greer et al., 1991a,b; Romero et al., 1997). In this study, STR typing was successful in many STR loci by the detection of PCR product of short size, but it was unsuccessful in several loci when detection of PCR product of long size was attempted. Our experience was that the size of the detectable PCR product in STR loci was short when the period of formalin fixation was long. When the formalin fixation was more than one week, the STR loci of more than 200 bp became undetectable. There are some reports in regard to such phenomena (Greer et al., 1991a,b; Yamada et al., 1994). A simple formalin dilution solution has an acidity of about pH 4, but buffered formalin is adjusted to pH 7.4-7.6 by a phosphate buffer. Neutral pH can prevent DNA fragmentation by formalin fixation. In this case, the tissue samples were stored in 20% buffered formalin for 2 weeks due to the “golden week”, a long holiday in Japan. It was higher than the standard concentration (10%) and longer than the usual period (3 to 5 days). However, it is thought that STR typing was successful because the fixed solution was not a simple formalin solution but a buffered formalin solution. Therefore, the tissue samples should be fixed in buffered formalin solution when considering DNA typing after histological diagnosis.

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However, this DNA contained PCR-amplifiable and unamplifiable DNA. Moreover, the grade of digestion of protein-protein and protein-nucleic acid crosslinks influences the result of STR typing. Therefore, it is preferable that PCR amplification is carried out at various concentrations in the case of formalin-fixed samples.

 Clinically, STRs can be used to study genetic alterations in tumors. A genetic deletion common to many types of cancer is referred to as the loss of heterozygosity (LOH). Numerous examples of LOH in cancer have been described (Silva et al., 1997, 1998; Pai et al., 2002; Poetsch et al., 2004; Vauhkonen et al., 2004) and some have been mapped in areas located in close proximity to markers employed in human identity testing. Despite this fact, LOH has rarely been observed for STR loci commonly employed in forensic testing. Although we observed an allelic imbalance at the D8S1179 locus, this did not cause any difficulty in STR typing.

 Recently, multiplex PCR and minisequencing of SNPs have been studied for human identification (Sanchez et al., 2003, 2006). Gilbert et al. demonstrated that multiplex PCR with minisequencing (MPMS) will prove useful some day when FFPE materials are analyzed (Gilbert et al., 2007b). In forensic science, the AmpF/STR® MiniFiler™ PCR Amplification Kit (Applied Biosystems) has become commercially available for forensic samples (Butler et al., 2003; Chung et al., 2004; Coble and Butler, 2005; Hill et al., 2007). This amplification kit provides information on the 8 largest and most difficult to amplify loci in the AmpF/STR® Identifiler™ Kit and SGM Plus®. With amplicon sizes of less than 270 bp, MiniFiler™ is optimized for use with the most difficult types of samples, including those which are degraded and contain inhibitors. This kit may be useful in such formalin-fixed samples to recover the larger loci, which cannot be amplified or which drop out.

 Over 2 years of follow-up after the DNA typing, this patient has survived without malignancy. Clinical tissue samples are not usually employed in forensic casework. However, personal identification using DNA typing in forensic science proved very useful for clinical medical treatment. We think that such forensic DNA typing may be developed in the future. Furthermore, there is another issue in forensic casework for clinical cases. Usually, DNA typing costs 100,000 yen ($800) in Japan, so we are confronted with the problem of expenses. In Japan, when it is suspected that a patient's sample may have been mistaken for another patient's, the budget for such a problem is not appropriated. In future, it should be decided who bears the expense for personal identification in such clinical cases.

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