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ORIGINAL ARTICLE

Authentication of newly established human esophageal squamous cell carcinoma cell line (YM-1) using short tandem repeat (STR) profiling method

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Abstract Cross-contamination during or early after establishment of a new cell line could result in the worldwide spread of a misidentified cell line. Therefore, newly established cell lines need to be authenticated by a reference standard method. This study was conducted to investigate the authenticity of a newly established epithelial cell line of human esophageal squamous cell carcinoma (ESCC) called YM-1 using short tandem repeat (STR) DNA profiling method. Primary human ESCC epithelial cells were cultured from the fresh tumor tissue of an adult female patient. Growth characteristics and epithelial originality of YM-1 cells were studied. Genomic DNA was isolated from YM-1 cells harvested at passage 22 and ESCC donor tumor sample on two different days to prevent probable DNA contamination. STR profiling was performed using AmpFℓSTR® Identifiler® Plus PCR Amplification Kit. To address whether YM-1 cells undergo genetic alteration as the passage number increases, STR profiling was performed again on harvested cells at passage 51. YM-1 cells grew as a monolayer with a population doubling time of 40.66 h. Epithelial originality of YM-1 cells was confirmed using ICC/IF staining of cytokeratins AE1/AE3. The STR profile of the ESCC donor tumor sample was the same with YM-1 cells at passage 22. However, STR profile of the donor tumor sample showed an off-ladder (OL) allele in their D7S820 locus. Also, re-profiling of YM-1 cells at passage 51 showed a loss of heterozygosity (LOH) at D18S51 locus. This suggests that long-term culture of cell lines may alter their DNA profile. Comparison of the DNA fingerprinting results in DSMZ, and ATCC STR profiling databases confirmed unique identity of YM-1 cell line. This study provides an easy, fast, and reliable procedure for authentication of newly established cell lines, which helps in preventing the spread of misidentified cells and improving the reproducibility and validity of experiments, consequently.

Keywords STR profiling · Authentication · Cross-contamination · Human ESCC · Newly established · YM-1 cell line

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Introduction

Establishment and authentication of continuous cell lines originated from human tumor tissues may be useful in studying the cellular and molecular characteristics of the same tumor [1] and minimizing the use of animal models [2]. However, numerous warnings have emerged from the literature concerning appropriate use of cell lines as model systems [3, 4]. Cell line misidentification is one of the most serious and persistent problems which often originates from cross-contamination [5]. An estimated 36 % of cell lines used in experiments have been misidentified or cross-contaminated with other cell lines [6–8]. Scientific misrepresentations and invalid results are the most irrecoverable outcomes of using misidentified cell lines during experimental studies.

[6]. Cross-contamination may happen under several conditions, including poorly controlled manipulation, sharing culture media and reagents, simultaneous manipulation of multiple cultures, and mislabeling a flask or ampoule [5, 9, 10] (ASN-0002). In addition, mitotically inactivated feeder cells, xenografting and conditioned media may carry contaminating cells if not properly eliminated [11].

Awareness of misidentification dates back to the 1950s [10]. Stanley Gartler, for the first time, introduced the concept of biochemical polymorphisms to distinguish human cell lines on the basis of their isozyme expression [12]. DNA fingerprinting for cell authentication purposes, which was based on polymorphisms in DNA sequences at given loci, was firstly employed by Masters et al. and Thacker et al. [13, 14]. Sensitivity of DNA fingerprinting for cell authentication was further improved by PCR-based short tandem repeat (STR) typing [15]. STR profiling, which currently serves as an international reference standard for human cell line authentication, is a rapid, reproducible, and standardized PCR-based method [8]. Using this method, various polymorphic STR loci are amplified, and the resulting PCR products are then resolved by capillary electrophoresis [6]. The sized PCR products are then converted into alleles with comparison to the standard allelic ladders, and the assigned alleles are then converted into numeric values which are used to create a baseline profile [16]. Comparison of the STR profile with a reference standard database determines whether or not a cell line is misidentified.

Cross-contamination of newly established cell lines could result in the worldwide spread of misidentified cell lines [7, 17]. To our knowledge, there have been no published studies that recommend the authentication of newly established cell lines prior to entering cell line collections and general use by other researchers. This study was aimed to investigate the authenticity of newly established ESCC cell line called YM-1 and describe that authentication of new cell lines can help prevent the worldwide spread of a misidentified cell line, which, in turn, prevents obtaining of misleading results.

Material and methods

Newly established YM-1 cell line and culture condition

Tumor specimen

A fresh tumor specimen was derived from a 50-year-old Iranian woman (Turkmen race) with esophageal squamous cell carcinoma. The specimen was resected from middle intra-thoracic esophagus (30 cm from incisors) prior to chemotherapy. The study was approved by the Ethics Committee of Golestan University of Medical Sciences and written informed consent was obtained from the patient.

A primary culture

Immediately after resection, tumor specimen was placed in a 15-ml conical centrifuge tube containing 5 ml of ice-cold transport medium and transferred to the lab (≈30 min). A small piece of tumor tissue (approximately 0.5 cm^3) was fixed in 10 % formalin for 24 h and processed for hematoxylin and eosin (H&E) staining. Also, a small piece of tumor tissue (approximately 0.5 cm^3) was frozen at −70 °C for DNA extraction.

Tumor tissue was washed five times in a Petri dish containing fresh, sterile ice-cold DBSS medium and minced by cross-cutting with two scalpels into small pieces as by size of 1 mm^3. The tissue pieces were then transferred into a 50-ml sterile conical centrifuge tube containing 5 ml Trypsin-EDTA

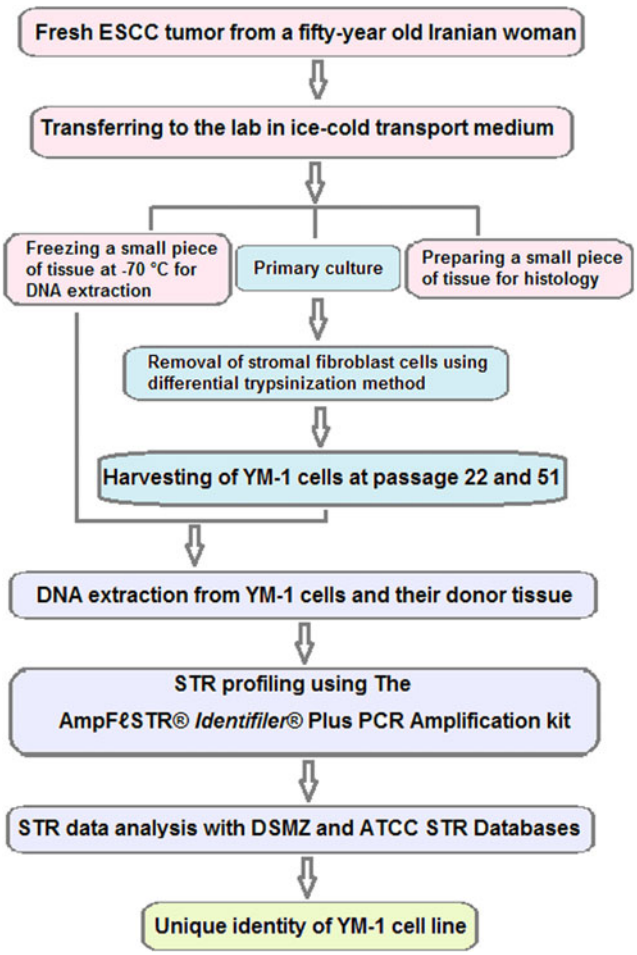

Fig. 1 STR profiling process. After transferring the tumor sample to the cell culture laboratory, primary cultures were performed. Two small pieces of tumor sample were also stored for DNA extraction and pathology examination. Then, STR profiling was performed, and the data were analyzed by comparing with DSMZ and ATCC databases. For more details, see the text. *ESCC* esophageal squamous cell carcinoma, *STR* short tandem repeat, *DSMZ* Deutsche Sammlung von Mikroorganismen und Zellkulturen, *ATCC* American type culture collection

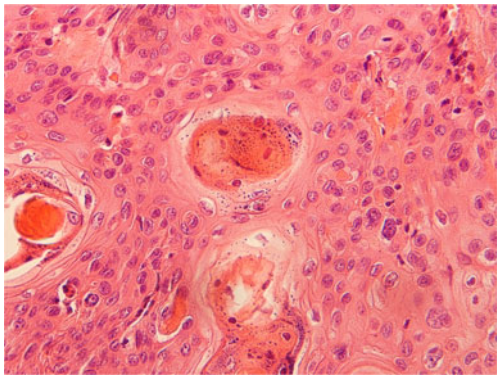

Fig. 3 Well-differentiated squamous cell carcinoma of esophagus. The tumor consists of large cells and shows prominent keratinization. ×200

(0.25 %) at room temperature. Supernatant was collected (without centrifugation) every 15 min and replaced by Trypsin-EDTA (0.25 %). The process was repeated several times until the tissue was completely dispersed. The clump-free cell suspension was decanted into another 50-ml sterile conical centrifuge tube containing growth medium (RPMI with 10 % FBS). The suspension was then centrifuged for 3 min at 1000 rpm. The supernatant was discarded, and the cells were re-suspended in RPMI 1640 medium supplemented with 10 % FBS, penicillin G (100 U/ml), streptomycin (100 μg/ml), gentamicin (2.6 μg/ml), and fungizone (2.5 μg/μl). Finally, the cells were seeded in 25-cm^2 culture flasks and placed in a CO2 incubator at 37 °C. The flasks were examined daily. One third of the medium in each flask was replaced every 3 to 5 days by fresh culture medium. Stromal fibroblast cells were diminished using the differential trypsinization method [18].

Growth characteristics of YM-1 cell line

Approximately, 3×10^4 YM-1 cells of passage 51 were seeded into each well of 35-mm six-well dishes with 3 ml of growth medium that was replaced every 2 days. The number of viable cells was determined by the dye exclusion method every 24 h for 10 days using the trypan blue staining. The growth curve was plotted, and the doubling time was calculated from the data obtained from the logarithmic phase of the growth curve (Roth V. 2006 <http://www.doubling-time.com/compute.php>).

Immunocytochemistry/immunofluorescence (ICC/IF) staining of cytokeratins

Expression of cytokeratins was examined by ICC/IF staining of YM-1 cells at passage 50 with pan-Cytokeratin (AE1/AE3) (Santa Cruz Biotechnology Cat# sc-81714). YM-1 cells were fixed with 4 % paraformaldehyde in PBS, permeabilized with 0.1 % Triton X-100 in PBS for 15 min, and blocked with 1 % BSA in PBS for 45 min at room temperature. Samples were incubated with primary antibody (1/100 in PBS) overnight at 4 °C. Sheep Anti Mouse Ig-Human Ads- FITC conjugated (SINA BIOTECH Cat#SB-029921) (IgG polyclonal; 1/50) was used as the secondary antibody. Cells were incubated for 10 min in the diluted DAPI (1 μg/ml in PBS) as a

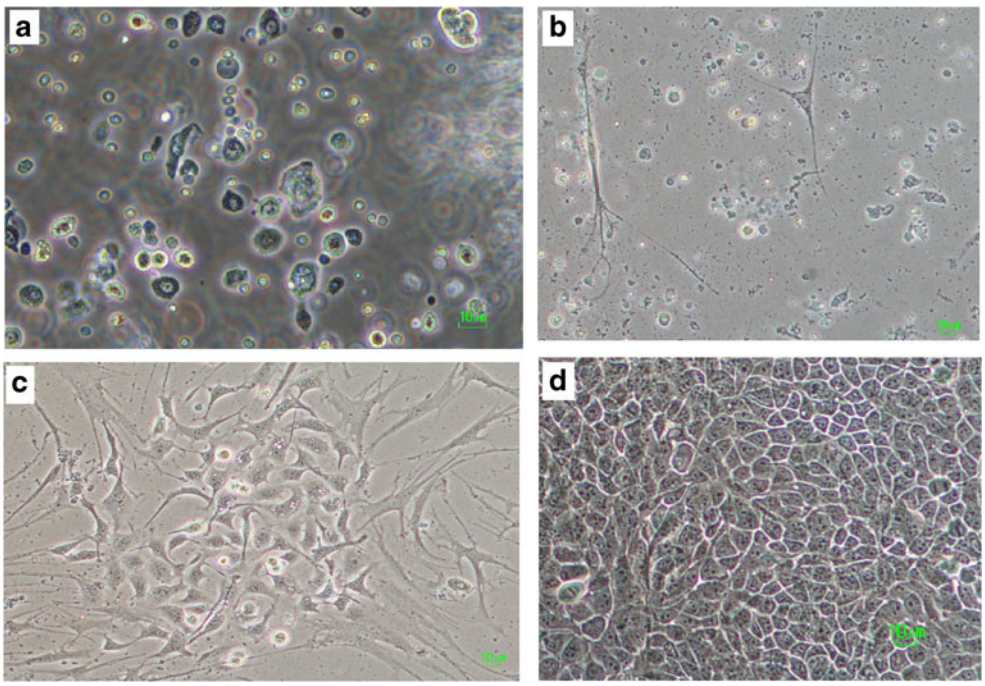

Fig. 4 Morphology of primary cells. **a** Cells after dissociation of human ESCC tumor. **b** Nervous-like cells. **c** Epithelial-like cells surrounded by stromal fibroblast cells. **d** Epithelial cells after passage 22. ×100

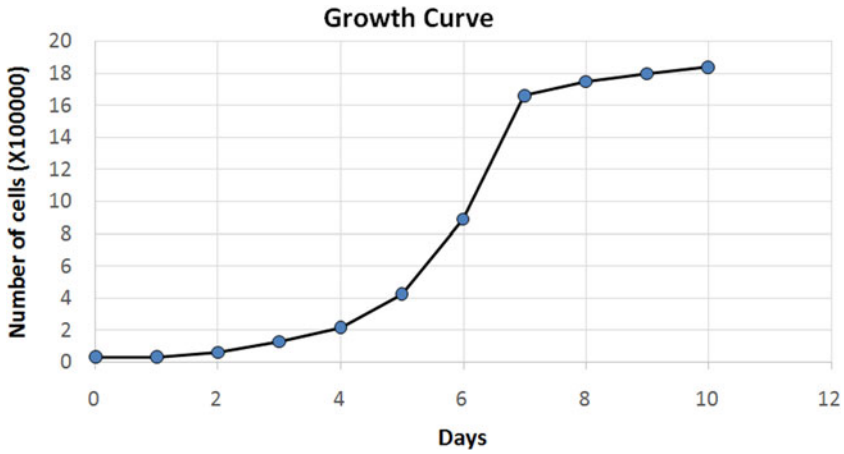

Fig. 2 Growth curve of YM-1 cells at the 51st passage

counterstain, before visualization by inverted fluorescent microscope (Nikon Eclipse Ti).

DNA extraction

Genomic DNA of YM-1 cells (5×10^5) at passage 22 and their donor tissue was purified using the High Pure PCR Template Preparation Kit (Roche, Germany) in different days to prevent cross-contamination according to the manufacturer's instructions. DNA concentration was determined by Picodrop UV/Vis Spectrophotometer (Picodrop Ltd, UK) at 260 nm. One nanogram of DNA of each sample was used for STR profiling.

STR profiling

To ensure that our cell line was authentic, STR profiling of both YM-1 cell line and donor tissue was performed and compared. Briefly, STR profiling procedure were as follows: 15 tetranucleotide repeat loci (D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818, and FGA) and the amelogenin gender-determining marker were amplified using the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit (Applied Biosystems, USA) according to the manufacturer's instructions. Electrophoretic analysis was carried out using ABI PRISM1 310 Genetic Analyzer (Applied Biosystems, USA). After electrophoresis, the data were analyzed by the Gene Mapper® ID-X Software v1.0 (Applied Biosystems, USA) to categorize peaks according to their size in relation to an internal standard allelic ladder.

Re-STR profiling of YM-1 cell line at passage 51

To address whether YM-1 cells undergo genetic alteration as the passage number increases, STR profiling was implemented again using the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit on harvested cells at passage 51.

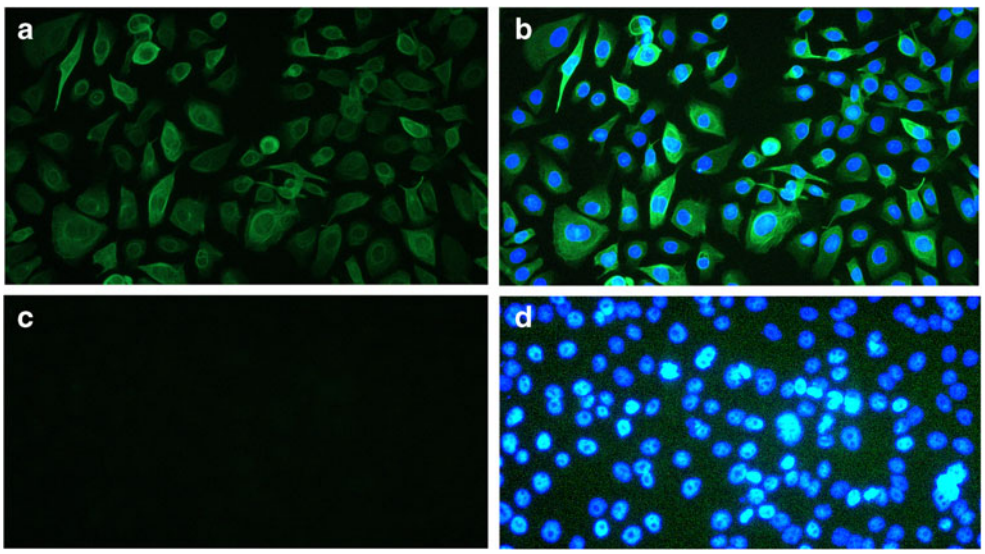

Fig. 5 ICC/IF staining of cytokeratins. The YM-1 cells were strongly positive for cytokeratins AE1/AE3 by ICC/IF. Anti-AE1/AE3 positive (**a**), anti-AE1/AE3 positive+DAPI (**b**), negative control, anti-AE1/AE3 negative (**c**), and anti-AE1/AE3 negative+DAPI (**d**)

Data analysis

STR data were analyzed using two online databases, a German Collection of Microorganisms and Cell Cultures, DSMZ, and ATCC STR database. After registration, our STR data were entered to be matched with authenticated cell lines listed in these databases.

Results

The whole process is summarized in Fig. 1. In this study, the YM-1 cell line was established from a female patient with a well-differentiated squamous cell carcinoma (Fig. 3) who had not received chemotherapy, previously. Histological diagnosis was determined on the basis of microscopic features of carcinoma cells. Fibroblast cells appeared on the third day. Besides fibroblast cells, abnormal cells were observed on the early days of primary cell culture. Some of these cells were nervous-like which were gradually eliminated (Fig. 4). Initial epithelial-like cells were observed on the 11th day of initial plating, but initial cell passage was performed 3 weeks later. Stromal fibroblasts were eliminated by differential trypsinization [19].

The YM-1 cells grew as an adherent monolayer with epithelial features and nuclear abnormalities. These cells are now (August 2015) at passage 53 and have been growing continuously for more than 14 months. The growth curve of these

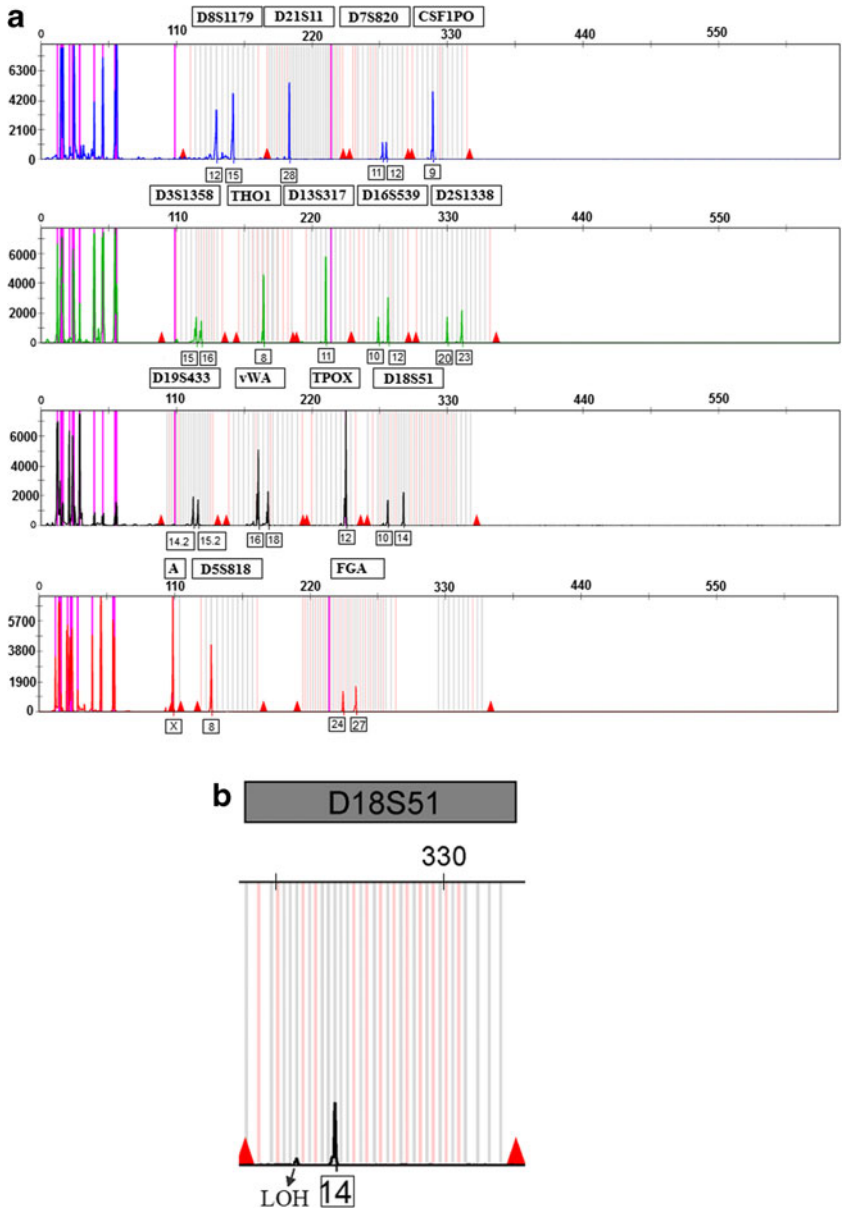

Fig. 6 The STR profile of YM-1 cell line. The figure shows the 16-locus multiplex fingerprint of YM-1 cell line at passage 22 (**a**) and the presence of LOH at STR locus D18S51 of these cells at passage 51 (**b**). The LOH data indicate deletion (*arrow*) of the allele 10, resulting in apparent homozygosity. STR loci are indicated in *boxes* above electropherogram; numbers of repeat units are indicated *below* the peaks. *STR* short tandem repeat, *LOH* loss of heterozygosity, *A* amelogenin

cells is illustrated in Fig. 2. Their population doubling time was 40.66 h. The YM-1 cells were strongly positive of cytokeratins AE1/AE3 at passage 50 by ICC/IF staining. This confirmed the epithelial origin of YM-1 cells. Negative controls were prepared by substituting the blocking buffer (1 % BSA in PBS solution) with primary antibody; and no detectable staining was evident (Fig. 5c).

The DNA profiles of YM-1 cell line are shown in Fig. 6. The genotypes of eight core loci were D5S818 (8, 8), D13S317 (11, 11), D7S820 (11, 12), D16S539 (10, 12), vWA (16, 18), TH01 (8, 8), TPOX (12, 12), and CSF1PO (9, 9) (Fig. 6a).

The genotypes of seven additional loci included in the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit were the following: D8S1179 (12, 15), D21S11 (28, 28), D3S1358 (15, 16), D2S1338 (20, 23), D19S433 (14.2, 15.2), D18S51 (10, 14), and FGA (24, 27) (Fig. 6a). When the YM-1 cell line and donor STR profiles were compared, both had identical profiles except for D7S820 locus. The donor STR profile showed the off-ladder allele in D7S820 locus (11, OL genotype). Re-STR profiling of YM-1 cell line was performed at passage 51, and genotype of each locus was determined. The STR profiles of both passages were identical. Loss of heterozygosity (LOH) was observed in D18S51 locus (Fig. 6b). The STR profile and chromosomal localization of each of the investigated loci are summarized in Table 1.

The gender of both donors and our YM-1 cell line were similar and that was consistent with the female gender of our patient. STR profiles were compared using DSMZ and ATCC databases to find the closest match to our samples. Both databases employed a minimum set of loci, eight STR loci plus amelogenin. Online STR matching analysis with DSMZ and ATCC databases showed that YM-1 cell line was not misidentified.

Discussion

The morphology of the YM-1 cells was similar to previously established cell lines derived from esophageal squamous cell carcinoma (e.g., HKESC-1 and KYSE-220 cell lines) [20, 21]. However, ESCC cell lines that have been established, so far, are different from each other in terms of doubling time and growth characteristics. To address whether YM-1 cells are of

Table 1 The STR profile of YM-1 cells at passages 22 and 51, and their donor tissue

Locus	Chromosomal location	Genotypes of samples		
		Tumor tissue	YM-1 cells at passage 22	YM-1 cells at passage 51
Eight core loci				
D5S818	5q21-31	8, 8	8, 8	8, 8
D7S820	7q11.21-22	11, OL	11, 12	11, 12
D13S317	13q22-31	11, 11	11, 11	11, 11
D16S539	16q24-qter	10, 12	10, 12	10, 12
CSF1PO	5q33.3-34	9, 9	9, 9	9, 9
TH01	11p15.5	8, 8	8, 8	8, 8
vWA	12p12-pter	16, 18	16, 18	16, 18
TPOX	2p23-2per	12, 12	12, 12	12, 12
Seven additional loci				
D2S1338	2q35-37.1	20, 23	20, 23	20, 23
D3S1358	3p	15, 16	15, 16	15, 16
D8S1179	8p	12, 15	12, 15	12, 15
D18S51	18q21.3	10, 14	10, 14	LOH, 14
D19S433	19q12-13.1	14.2, 15.2	14.2, 15.2	14.2, 15.2
D21S11	21q11.2-q21	28, 28	28, 28	28, 28
FGA	4q28	24, 27	24, 27	24, 27
Amelogenin	X: p22.1-22.3			
	X, X	X, X	X, X	
	Y: p11.2			

The table summarizes the chromosomal localization of each of the investigated loci (AmpFlSTR® Identifiler® Plus PCR Amplification Kit User Guide, page 3). YM-1 cells after passage 51 show loss of heterozygosity (LOH) in D18S51 locus.

STR short tandem repeat, *OL* off-ladder, *LOH* loss of heterozygosity

epithelial origin, expression of cytokeratins was examined by ICC/IF staining of YM-1 cells at passage 50 with pan-Cytokeratin (AE1/AE3). AE1/AE3 is a broad spectrum anti pan-cytokeratin antibody cocktail, which distinguishes epithelial tumors (e.g., ESCC) from non-epithelial tumors (http://www.novusbio.com/pan-Cytokeratin-Antibody-AE1–AE3_NBP2-29429.html). This antibody has been used to confirm the epithelial lineage of esophageal cancer-derived cell lines, previously [20, 22, 23]. The YM-1 cells were strongly positive of cytokeratins AE1/AE3 by ICC/IF (Fig. 5). Therefore, epithelial origin of the YM-1 cells was confirmed.

Recent studies reported that cross-contamination of mammalian cell lines has formed a major problem in the scientific community and led to the emergence of false scientific data [1, 24, 25]. The first report of cross-contamination of esophageal cancer cell lines goes back to 1988 by Paul D. van Helden et al. [26]. They showed that esophageal HCu cell lines have became cross-contaminated with other cells [26]. Also, they showed the cell lines HCu 10, 18, 33, 37, and 39 were genetically identical and were, in fact, subcultures of the same esophageal cancer cell line [26]. The results of Boonstra et al. suggested that the TE-7 cell line was not an esophageal adenocarcinoma, but a squamous cell carcinoma cell line [1]. Recently, they showed that three widely used esophageal cancer cell lines were, in fact, derived from other tumor types [17]. Primary cell cultures undergo a prolonged period of time before the emergence of an immortalized cell population, and this is a time, even if a single cell introduced from a separate cell line would rapidly outgrow the other, leading to a pure culture of the contaminating cells in four or five passages [10, 11] (ASN-0002). Therefore, cross-contamination during or early after establishment of a new cell line, especially, may result in the worldwide spread of a misidentified cell line [7, 17].

In this study, the newly established YM-1 cell line, originated from esophageal squamous cell carcinoma, was evaluated for authenticity using the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit. This kit has been designated for forensic application, and all 13 of the required loci for the combined DNA index system (CODIS) loci are included in this kit [27]. Increasing the number of STR loci included in this kit will increase the probability that the STR profile of YM-1 cell line is correctly authenticated as arising from a related donor. All the loci except the amelogenin gene in this kit are true tetranucleotide repeats. The length differences among alleles of a particular locus result from differences in the number of 4-nt repeat units.

No difference was found between the YM-1 cell line and donor STR profiles except for D7S820 locus. The STR profile of the donor showed the off-ladder or microvariant allele in D7S820 locus (11, OL genotype). Allelic ladders represent the most common alleles at each locus. Alleles with size outside allele categories represented in the ladder are known as off-ladder (OL) alleles [16]. To answer the question whether YM-1 cells undergo genetic alteration when passage number increases, STR profiling was repeated in cells that were harvested at passage 51. Surprisingly, allele 10 of the D18S51 locus was lost (Fig. 6b). The mechanism of the loss of this allele is unclear; however, it seems that when passage numbers increase, it results in genetic alteration. The results of Parson et al. suggest that long-term culture of cell lines may alter their DNA fingerprints [6, 8]. It is accepted within the forensic science that STR profiling of tumor samples and especially tumor-derived cell lines is not stable because of loss of heterozygosity and microsatellite instability [8, 28].

In AmpFℓSTR® Identifiler® Plus PCR Amplification Kit, the primers for the amelogenin locus flank a six-nucleotide deletion within intron 1 of the AMELX. Amplification results in 107-nt and 113-nt products from the X and Y chromosomes, respectively. After amplification of the AMEL locus, only the related picks of shorter AMELX fragments (107-nt) were observed and this means that both DNA donors are individuals with only X chromosomes, who will usually display a female phenotype. This finding was in relevant with the female gender of our patient.

The DSMZ, together with other databases such as the ATCC, has generated large comprehensive international reference STR profile database for human cell lines. Registered users simply can input their own cell line STR data to retrieve best matches with authenticated cell lines listed on the DSMZ and ATCC databases. These STR profile databases use eight STR loci, plus the amelogenin marker, recommended by ANSI/ATCC [29]. In this study, for STR matching analysis using DSMZ, the evaluation value of 0.6 was entered. STR matching indicated that the YM-1 cell line has a ≤60 % matching. Also, cell lines showed that shared alleles were not used in our laboratory during the establishment of our cell line. A comparison of our STR profile with the ATCC database showed also that YM-1 cell line had less than 56 % matched with cell lines of this database. Most studies agree that 80 % similarity is an appropriate threshold for declaring a match when comparing cell line profiles [6, 30].

Therefore, the YM-1 cell line is an authentic ESCC cell line and can be used as in vitro model in medical research. We suggest that all new established cell lines will be authenticated by the reference standard method such as STR typing in characterization process. Authentication at this process will prevent the spreading of the misidentified cells and help the reproducibility and validity of experiments. This study provides an easy and reliable way for authenticating newly established cell lines.

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Compliance with ethical standards The study was approved by the Ethics Committee of Golestan University of Medical Sciences and written informed consent was obtained from the patient.

Conflicts of interest None

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