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Applying Subtractive Hybridization Technique to Enrich and Amplify Tumor-Specific Transcripts of Esophageal Squamous Cell Carcinoma

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Abstract Subtractive hybridization (SH) as an efficient and powerful approach can be applied to isolate differentially expressed transcripts as well as detect of involved mRNAs in various cellular processes, particularly diseases and malignancies. This procedure leads to the enrichment of specific low copy transcripts of tumor cells. Having developed a new approach for SH to isolate tumor specific transcripts, we facilitated discovery of uniquely expressed genes in esophageal squamous cell carcinoma (ESCC). Total RNA was extracted from the fresh tumoral and their adjacent normal tissues, and purified using the Switch Mechanism At the 5′ end of Reverse Transcript (SMART) method. Following cDNA synthesis of normal mRNAs using magnetic beads, it was hybridized with tumor mRNAs. To enhance efficiency of subtraction, hybridization was repeated three rounds. Finally, amplification of subtracted tumor-specific transcripts was carried out using in vitro transcription. The subtracted tumoral mRNAs was analyzed quantitatively using real-time PCR for both tumor-specific and housekeeping genes. The subtracted mRNA was confirmed as tumor-specific mRNA pool using RT-PCR and quantitative real-time PCR assessment. The elevated level of tumor-specific transcripts such as MAGE-A4 and CD44 as well as declined copy number of housekeeping genes such as GAPDH, β actin and β2-microglobulin, were confirmed in subtracted tumoral mRNA. The presence of tumor genes was confirmed after the SH procedure. The designed SH method in combination with SMART technique can isolate and amplify high quality tumor-specific transcripts even from small amount of tumor tissues. Removal of common transcripts from the extracted tumoral mRNAs using SH, leads to the enrichment of tumor-specific transcripts. The isolated transcripts are of interest because of their probable roles in ESCC progression and development. In addition, these tumor-specific mRNAs can be applied for future vaccine cancer studies.

Keywords Subtractive hybridization · ESCC. Enrichment · Tumor-specific transcripts · SMART

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Introduction

Esophageal squamous cell carcinoma (ESCC) is the most prevalent cancer in Iran and the sixth leading cause of cancer-related deaths worldwide [1, 2]. The majority of ESCC patients are principally recognized at advanced stages of the disease due to asymptomatic progression and late diagnosis of malignancy [3]. The conventional cancer treatment methods including surgery, chemo-and radiotherapy were not sufficient to improve outcome of the disease, leading very low 5-year survival of patients nearly 20 % [4–6]. Accordingly, new and effective therapeutic strategies against different cancer types are required to increase survival rate of patients especially in early diagnosed cases [7]. In line with this requirement, immunotherapy is one of the most attractive therapeutic strategies for cancer, which is designed based on tumor-specific antigens [8]. Current cancer immunotherapeutic approaches attempt to enhance effectively the host's immune system, assisting identification and removal of tumor cells. In addition, these approaches can modulate the molecular processes involved in cancer progression and

development. Among different types of immunotherapy, immunotherapeutic vaccines are now being tested to treat a variety of cancers. These vaccines are designed based on tumor-specific antigens (which can excite immune response) for improving immune system to either eliminate tumor cells or arrest their recurrence after conventional cancer therapies [9, 10]. In such scenario, not only choose of appropriate tumor-specific antigens as targets for immunotherapy is critical, but also proper presentation of these antigens by antigen presenting cells is important to inhibit immune evasion of tumor cells [11]. Selection, isolation and enrichment of suitable and defined tumor-specific antigens are the main steps of cancer immunotherapy. Although antigens can be used as DNA, RNA and polypeptide, numerous studies have applied tumor antigens mRNA as cancer vaccine [12–15]. A number of known and unsuspected gens may be contributed to progress and development of cancer. There are various methods to evaluate differential gene expression in cancer; however, it is necessary to use an approach for the isolation of the novel involved mRNA sequences in the disease. Selection of the appropriate technique for isolation and detection of such mRNA sequences depends on available materials and desirable goal. One of the problems associated with ESCC immunotherapy using total tumor mRNA-loaded dendritic cells (DCs) were minor cytotoxicity response and probable autoimmune-response against normal RNA-loaded DCs and probable detrimental effects on noncancerous tissues [4, 16]. Since self-antigens in immunotherapy increase the risk of autoimmunity diseases, subtractive hybridization (SH) which enrich the tumor-specific mRNAs can improve this problem, effectively [17]. This study is focused on the usage of SH technique for isolation of tumor-specific transcripts associated with ESCC; even without any prior knowledge of cancer-associated genes identification. The SH is a powerful, sensitive and effective technique which allows isolation and characterization of nucleic acids sequences. The advantages of this approach are comparing the expression patterns of various rare transcripts, discovery of unknown genes, explanation of the molecular mechanisms as well as subtraction of common sequences between two different populations from two closely related sources [18, 19]. Fundamentally, this technique consists of the several basic steps consisting of preparation of the driver and target, hybridization, subtraction as well as isolation of the remaining target RNAs. In the subtractive enrichment procedure, the tested population contains the target sequences which are enriched through hybridization with an excessive amount of the driver or control mRNA population. The common sequences of target and driver populations are hybridized with each other and the enrichment of the target sequences is performed by elimination of driver and common sequences. In the subtraction step, all of tester-driver hybrids and excess driver sequences are removed; and finally the remaining target sequences are enriched as the target specific sequences. After the subtraction step, the remained tumor-specific sequences can be utilized for a variety of applications such as RNA next generation sequencing, making a library enriched of target specific sequences and genetic engineering research [20, 21]. It is noteworthy that preparation of RNAs, cDNA generation and finally isolation of target-specific mRNA from the solutions are performed using magnetic beads. This approach is based on the poly(A) tail of mRNAs which bind to magnetic beads which are coated with oligo(dT). In this process, the purified mRNA is directly used for reverse transcription and cDNA generation on beads [22].

In this study, we aimed to enrich the tumoral-specific transcripts via the subtraction of common normal sequences. To test this hypothesis, tumor-specific mRNAs of ESCC tissues were isolated using novel strategy for preparing subtractive hybridization approach and increase the impressive concentration of these target sequences. Meanwhile, we comparatively investigated the expression levels of known genes before and after subtractive hybridization to identify truly tumor specific sequences.

Materials and Methods

Tissue Specimen Preparation

Fresh tumors and their margin non-tumor tissues were obtained from five ESCC patients (Table 1) during esophagectomy at Omid Oncology Hospital of Mashhad University of

Table 1 Clinicopathological characteristics of patients

Patient	Age	Sex	Tumor Location	Tumor Grade	TNM Classification
Patient 1	48	Male	Lower	P.D.	T3N1M0
Patient 2	51	Female	Upper	W.D.	T3N0M3
Patient 3	75	Male	Middle	P.D.	T3N0M2
Patient 4	40	Female	Middle	M.D.	T3N0M2
Patient 5	47	Male	Lower	M.D.	T1N0M2

N0: No regional lymph node metastasis; N1: Metastasis in 1 to 2 regional lymph nodes; N2: Metastasis in 3 to 6 regional lymph nodes; N3: Metastasis in 7 or more regional lymph nodes

WD Well differentiated, *MD* Moderately differentiated, *PD* Poorly differentiated

Medical Sciences (MUMS), Iran. The samples were immediately transferred into the RNAlater solution (Qiagen, Hilden, Germany) to stabilize the RNA and stored at −20 °C. None of the patients received other alternative pre-operative treatment such as chemo- or radiotherapy. The normal and tumor tissues were approved histologically by expert pathologists. Tumor samples were microscopically checked to confirm that all the enclosed specimens contain more than 70 % tumor cells. The study protocols were approved by the research Ethics Committee of MUMS and all the patients written their informed consent before the surgery.

Total RNA Extraction

Total RNA of tissues was isolated from fresh-frozen tumor and the corresponding margin normal tissues using RNeasy Mini Kit (Qiagen, Hilden, Germany), as described before [23, 24]. The quality and quantity of extracted RNAs were analyzed using gel electrophoresis and spectrophotometery, respectively.

Subtractive Hybridization

Isolation of tumor-specific transcripts of ESCC tissues were performed using SH method. Briefly, this procedure includes isolation, amplification and preparation of both normal and tumor mRNAs, followed by cDNA synthesis from normal mRNA as driver on the magnetic beads. Afterward, the normal cDNA was hybridizes with prepared mRNA from tumor tissues as target. The subtracted tumoral mRNA obtained after three rounds of hybridization and subtraction. The descriptive protocol includes five steps:

Step 1- SH. Isolation and amplification of both of normal and tumor mRNA

Isolation, amplification and purification of mRNA were performed according to the manufacturer's instructions, as described before [25].

Step 2- SH. Preparation of normal (driver) mRNA

Preparation of normal tissue mRNA (driver or subtractor) was performed using Dynabeads oligo$(dT)_{25}$ (Dynabeads mRNA DIRECT Kit, Invitrogen) according to the subtractive hybridization protocol. Totally 1 μg of driver mRNA was equilibrated with the appropriate amount of DECP-water and incubated at 65 °C for 5 min. In addition, the appropriate quantity of Dynabeads oligo$(dT)_{25}$ was washed twice by Lysis/ Binding Buffer (BB), followed by mixing prepared mRNA and the beads/ BB (bead/BB + mRNA/DEPC water) and incubation on shakerfor15 min at room temperature to allow the polyA tail of the mRNA hybridize with Dynabeads oligo$(dT)_{25}$. Then the tube was placed into a magnetic separator (MS) before removing the supernatant. Finally, the beads/mRNA complexes were washed twice using Washing Buffer B, and re-inserted into the MS. Finally, the mRNA/beads sediment were ready for cDNA synthesis.

Step 3- SH. Preparation of immobilized subtractor cDNA on magnetic beads

The subtractor cDNA was synthesized directly on the magnetic beads using Dynabeads oligo$(dT)_{25}$ as primers by Maxima H Minus Reverse Transcriptase kit (Thermo scientific) according to the manufacturer's instructions. First, the driver mRNA from the previous step was washed three times with 1X reverse transcriptase buffer. Cleaned normal mRNA/Dynabeads oligo$(dT)_{25}$ primer was mixed with10 mM dNTP (Pars tous, Iran) and incubated at 65°Cfor 5 min and snap-cooled on ice. The reaction was followed by the addition of 5X RT buffer, Ribolock RNase inhibitor, Maxima H Minus Reverse Transcriptase (Thermo Scientifc, USA) to the mRNA solution. Afterward, the temperature was adjusted at 50 °C for 30 min followed by terminating the reaction at 85 °C for 5 min. The reaction was stopped by adding 2 mM EDTA and 95 °C heating for 3 min to denature the mRNA–cDNA hybrids. The mixed suspension was immediately placed into a MS to remove the supernatant containing mRNA (Eluted mRNA). Finally, the sediment subtractor cDNA/beads were washed three rounds with TE buffer (pH 8) and resuspended in TE and stored at 4 °C for subtraction hybridization step.

Step 4- SH. Preparation of tumor (target) mRNA

The amplified and purified tumoral mRNA which obtained from the SMART procedure was resuspended in hybridization buffer (4.5× SSPE, 0.1 % SDS).

Step 5- SH. Subtractive hybridization procedure

In order to obtain the subtracted tumor mRNA, we utilized one sample pair including tumor and normal mRNA of ESCC patients. First, both the target mRNA in hybridization buffer and subtractor cDNA/ bead in TE buffer were simultaneously heated at 68 °C for 3 min. The subtractor cDNA/ bead was immediately placed into a MS and supernatant was removed, then subtractor cDNA/ bead was resuspended in the target mRNA solution. Afterward, the hybridization mixture was incubated at 65–68 °C for 20–24 h in hybridization oven with gentle rotation to achieve the hybrids between the target mRNA and the subtractor cDNA/ bead mixture. Second, the reaction tube was transferred directly on ice and then placed into a MS. The supernatant containing the target-specific mRNA was transferred to a new RNase free tube and store on ice for the next round of hybridization. Then

subtractor cDNA/bead sediment was dissolved in DEPC-water and heated for 3 min at 95 °C to elute driver mRNA, and sediment was resuspended in TE buffer for further hybridization rounds. It is necessary to repeat steps of SH twice, in total 3 rounds of subtractive hybridization will be conducted. After third round; new Dynabeads oligo(dT)$_{25}$ was washed in hybridization buffer followed by isolation of the target-specific mRNA from the solution and transferring to the new beads. The solution was incubated for 10 min at room temperature under constant rolling. The tube containing mixed reaction was placed into MS removing the supernatant and sediment was washed once with washing buffer containing LiDS and twice with washing Buffer B. Then the reaction was followed by the addition of DEPC-water to the sediment and incubation at 65 °C for 2 min. Finally, the tube was immediately placed into a MS and the eluted mRNA containing subtracted tumoral mRNA was transferred to a new RNase-free tube and stored at −20 °C.

Subtracted Tumoral mRNA Amplification

The tumoral-specific mRNA from prior step was amplified using a SMART mRNA amplification kit (Clontech, USA), according to the manufacturer's instructions.

Analysis of the Subtraction Efficiency

Reverse Transcription (RT-PCR) Reaction

In order to confirm the quality of total normal/tumor mRNA and non-amplified/amplified subtracted tumoral mRNA, gene expression analysis of three housekeeping genes, including Glyceraldehyd 3-phosphat dehydrogenase (GAPDH), β actin and β2-microglobulinas positive control was performed using RT-PCR. Since overexpression of MAGE-A4 (Melanoma-associated antigen 4) as a specific biomarker and CD44 as a cancer stem cell marker was reported previously in ESCC tissues [26, 27], in next step we selected these genes to assess the efficiency of subtraction. The expression level of the genes was investigated by RT-PCR using the specific primer pairs presented in Table 2.

Real-Time PCR

To confirm the efficiency of subtraction, comparative real-time PCR was utilized to compare the presence of unsubtracted expressed genes (the housekeeping genes) before and after subtraction in triplicate reactions. The data were normalized for tumoral genes (CD44 and MAGE-A4) expression via the comparative threshold cycle method. The relative expression level of housekeeping genes in non-amplified and amplified subtracted tumoral mRNA sample was compared with total tumoral mRNA. The reverse transcription reaction was performed using oligo(dT) first-strand synthesis kit (Fermentas, Lithuania) in 20 μl reactions according to the manufacture's instruction. cDNA was amplified with primer sequence sets presented in Table 2 in Stratagene Mx3000P real-time thermocycler (Stratagene, La Jolla, CA) using SYBR green PCR Master Mix (Ampliqune, Denmark) The used thermal cycling program for CD44 was 10 min at 95 °C, followed by 40 cycles each of 95 °C for 15 s, 60 °C for 60 s, and 72 °C for 45 s. The applied thermal profile for MAGE-A4 was included 10 min at 95 °C as the initial denaturation step, followed by 40 cycles of 15 s at 95 °C, 30 s at 60 °C, and finally 45 s at 72 °C. Relative changes in presence of the GAPDH, β actin and β2-microglobulin transcripts in the subtracted and unsubtracted specimens was utilized for analysis of subtraction efficiency.

Results

Evaluation of Quality and Concentration of Obtained RNAs

Total RNA was extracted from normal (driver) and tumoral (target) tissue samples, followed by mRNA isolation from the

Table 2 The genes and primer sequences used in real-time PCR

Gene	Primer sequences(5′ → 3′)	Size (bp)
GAPDH	F: GGAAGGTGAAGGTCGGAGTCA R: GTCATTGATGGCAACAATATCCACT	101
βactin	F: AGAGCTACGAGCTGCCTGAC R: AGCACTGTGTTGGCGTACAG	184
β2-microglobulin	F: GGCACAACAGGTAGTAGGCG R: GCCACAGGAGCTTCTGACAC	170
CD44	F: TCCAACACCTCCCAGTATGACA R: GGCAGGTCTGTGACTGATGTACA	83
MAGE-A4	F: CCAAAGGCAACTTAAAGGTTCA R: CCGTGAAGACCAATGAGATCTC	108

total RNA using SMART mRNA amplification method. The quantity and quality of obtained RNAs were estimated using the determination of absorbance ratio and electrophoresis. For pure RNAs and subtracted tumor mRNA, A260/280 was in the range of 1.8 to 2. Moreover, using A260 and the Beer-Lambert law the concentration of RNAs was measured. Almost 1 μg of starting total RNA and subtracted tumor mRNA was used for purification and amplification of mRNA. As shown in Table 3, the *IVT* reaction was carried out for 12 h to obtain the additional amount of mRNA. The integrity of total RNA and mRNA samples was confirmed using 1.2 % agarose gel electrophoresis and confirmed the respective 18S and 28S ribosomal RNA bands and mRNA smear. As a result, good starting RNAs quality and purity was demonstrated as a critical element in successful results of SH.

Subtraction Efficiency

Analysis of the subtraction efficiency from each specimen was assessed for presence or absence of housekeeping (GAPDH, β actin, β2-microglobulin) and tumoral (CD44 and MAGE-A4) genes by RT-PCR and relative comparative real-time PCR. The presence of housekeeping genes in total normal/tumor mRNA and their absence in non-amplified/amplified subtracted tumoral mRNA samples were confirmed using RT-PCR approach (data not shown). This indicates that SH technique can remove housekeeping genes. In addition, all tumor mRNAs as well as non-amplified/amplified subtracted tumoral mRNAs showed the expression of CD44 and MAGE-A4 genes regarding the amplicons size on agarose gels as shown in Fig. 1. These results indicate that the SH method can isolate and enrich the specific tumoral genes in subtracted sample of ESCC. Finally, for corroborating the results obtained from RT-PCR, relative comparative gene expression analysis was performed on subtracted mRNA using the comparative real-time PCR method. The expression levels of housekeeping genes in non-amplified/amplified subtracted tumoral mRNA were compared to total tumor mRNA. The real-time PCR results demonstrated that relative expression levels of GAPDH, β actin, β2-microglobulin genes in the subtracted tumoral samples had 9.36, 13.7, and 7.22 negative fold change, respectively, in comparison with non-subtracted samples (Fig. 1). The results analysis illustrated that the copy number of these housekeeping genes were decreased in ESCC samples after subtraction approach especially, for β actin copy number.

Discussion

Enrichment of tumor-specific transcripts especially rare and unique mRNAs through elimination or declined level of either normal or housekeeping genes by SH approach from malignant tissues is urgently needed for new cancer therapeutic modalities. It has been previously shown that human DCs transfected with total tumor mRNA can induce cytotoxic effects of T cells in ESCC patients. In most types of such applied cancer vaccine in immunotherapy, the used total tumor mRNA is consisting of both tumor-specific and non-tumor transcripts. These non-tumor transcripts in cancer vaccine can lead to autoimmune response in treated patients. Accordingly, one of the critical issues in cancer immunotherapy strategies is the utilization of defined tumor-specific antigens which are restrictedly expressed in tumor cells to minimize induction of autoimmunity [4, 28]. This study is focused on introducing a new technical approach for isolation of target-specific transcripts based on the SH method in ESCC samples without any requirement to knowledge about the gene-specific sequences. Different tumor-specific biomarker is identified in ESCC which can be used in medical treatment approaches [29]. Accordingly the discovery and characterization of new tumor biomarkers which exclusively expressed in malignant tissues can help to design effective vaccine for therapeutic purposes [30].

Generally, the produced tumor-specific antigens by own tumor cells which are circulated through the body, can be defined as cancer biomarker to use in effective clinical cancer diagnosis [31]. It has been illustrated that own tumor antigens can induce the specific antitumor response against tumor cells, improving cancer immunotherapy [32]. The usage of such tumor antigens in form of mRNA can stimulate antitumor specific responses in patients with different malignancies including prostate, breast, colorectal cancers, renal cell carcinoma and adenocarcinoma [14, 28, 33–35]. Although some targeted antigens are often expressed by both noncancerous and cancerous cells, the aim of cancer immunotherapy using the specific or non-specific antigens is essentially elimination of cancer cells [36].

In this study, two types of genes were used for investigation of the subtraction efficiency including MAGE-A4 as a cancer-testis antigens and CD44 as a cancer stem cell marker. Cancer-testis antigens (CTAs), a subset of tumor associated antigens, are normally expressed in germ line and placenta but their aberrant expression is reported in a wide range of cancerous tissues as tumor-specific antigens [15, 37, 38]. MAGEA4 as

Table 3 Quantity of mRNA amplified in *IVT* reaction

Quantity of starting samples	Quantity of obtained mRNA by in vitro transcription (12 h)
1000 ng total RNA	18,000 ng
1000 ng subtracted tumor mRNA	10,000 ng

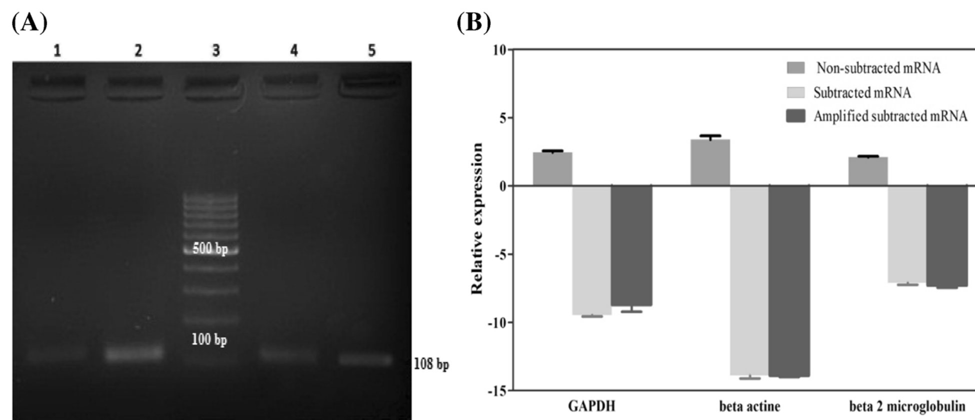

Fig. 1 Constructed subtractive hybridization. **a** Electrophoresis of unsubtracted and subtracted tumoral mRNA is shown. Lane 1: Unsubtracted driver mRNA, Lane 2: Unsubtracted tumor mRNA, Lane 3: Ladder, Lane 4: Subtracted tumor mRNA, Lane 5: Subtracted tumor mRNA of amplified (MAGE-A4). **b** Analysis of the subtraction efficiency. The relative expression levels of the housekeeping genes in the non-subtracted and subtracted samples (CD44 S)

one of CTAs is potentially introduced as an appropriate target for cancer vaccine strategies. The overexpression of MAGEA4 was significantly detected in more than90% of the ESCC patients introducing a special tumor-specific biomarker for advanced stages of ESCC [8]. CD44 as a cancer stem cell (CSC) marker plays a key role in tumor progression in various cancers including breast, oral, prostate, gastric, lung, colon malignancies as well as head and neck and ESCC [26]. In this study we investigated the presence or absence of MAGEA4 and CD44 genes in the target samples to analyze the subtraction efficiency. The usage of such unique antigens which their expression is absolutely restricted to tumor cells can be applied effectively for cancer vaccine immunotherapy while their toxicity against normal self-tissues is very low. Accordingly, the risk of autoimmunity sequelae in patients can be minimized after vaccination with tumor-specific antigens. In addition, the self-specific antigens can be aimed to remove and regulate the control mechanisms of both T and B cells which assist autoimmune tissue injury [36, 39]. Isolation and characterization of unique tumor biomarkers can help clinical cancer applications especially in ESCC patients [40]. Therefore, there is a most essential need to invent suitable methods for detection and isolation of interesting tissue specific antigens [41]. The identification of changes in gene expression pattern of tumor cells is severely required to develop new techniques to discover the involved genes in tumorigenesis process leading to more effective therapeutic methods [42, 43].

Use of methods with ability of differential comparison between tumor and normal cells to recognize the cancer specific genes, is an urgent need to improve our understanding of the molecular process of tumorigenesis. Different methods are developed for isolation and detection of differential gene expression in tumor cells compared to normal including subtractive hybridization, differential display, expressed sequence tags (ESTs) and serial analysis of gene expression (SAGE) as well as cDNA microarray technology [44]. This is the first report using subtractive hybridization method as an appropriate technique for isolation of the tumor-specific transcripts from ESCC tissue specimens as a rich source for tumor-specific antigen discovery. This approach was utilized to detect distinctions between the transcripts in two different cell populations (cancerous vs. normal). Additionally this approach eliminates the non-tumoral sequences from tumoral-specific transcripts, leading to consistent copy number of tumoral transcripts in tumor subtracted samples versus unsubtracted. In SH, two populations are required; the tumor mRNA as the target and the non-tumor cDNA as the driver. Briefly, the common sequences between two populations are hybridized to each other and any unhybridized driver molecules is subsequently eliminated in the subtraction step. The remaining target mRNA sequences are truly normalized and enriched as the tumor-specific sequences. This process is repeated two or three rounds by adding excess fresh driver to ensure lack of common transcripts in target sequences. These multiple rounds of hybridization are thoroughly required in order to remove the rare common sequences [44–46]. There are various methods for subtraction step such as hydroxyapatite, biotin and streptavidin, chemical cross linking and different immobilization approaches (cellulose, oligo (dT)-cellulose, oligo (dT)-latex, Dynabeads oligo (dT), or on a nitrocellulose membrane) [43]. Each of previous studies have applied a part of our designed stepwise method separately including the Dynabeads oligo (dT) for preparation of target and driver mRNAs, the generation of immobilized cDNA by reverse transcription (RT), target-driver hybrids and excess driver removal and also the separation of subtracted tumoral mRNAs [19, 22, 43, 44]. In the current study, immobilized cDNA driver was generated on magnetic beads using the oligo(dT) primer. Remarkably, this immobilization approach was

simple, practical and impressive procedure for solid phase generation of cDNA to apply in SH process. Furthermore, the magnetic bead separation method was used as a functional and particularly efficient technology to remove hybrids and target tumoral mRNA. We performed three rounds of subtraction to eliminate rare common sequences from the target sample completely. One of the substantial benefits of this technique is usage of small amounts of tumor tissue which prepare subsequently limited quantity of RNA source, although using the SMART mRNA amplification approach can result high quality mRNA as an input sample for downstream analysis such as SH [47]. The extracted total RNA from the available finite amount of starting tissues must be amplified and restricted to mRNA sequences before starting hybridization and subtraction approaches. Moreover, the obtained target tumor-specific mRNAs are regenerated and amplified after subtractive hybridization procedure applying in vitro transcription. The prepared target-specific mRNAs should be greatly analyzed using specific methods such as PCR, northern blotting, in situ hybridization and RNA sequencing to confirm that the obtained sequences are truly target-specific transcripts. The poly(A) reverse transcription polymerase chain reaction (RT-PCR) can analyze cellular function through assessment of gene transcription leading to reliable phenotype analysis of tumor cell [44]. In the present study, we also employed poly(A) RT-PCR for investigation of the SH efficiency, particularly for samples with a small amount of target mRNAs. Our results confirm the subtraction efficiency based on the presence of tumor-specific transcripts as well as absence of the housekeeping mRNAs in subtracted tumor specific sequences. A sufficient amount of amplified sense RNA was generated from limited quantity of input total RNA. The used procedure combines template-switching technology with T7 RNA polymerase transcription. Briefly, the SMART mRNA amplification procedure summarizes in three key steps. This approach begins with the synthesis of first-strand cDNA, followed by generation of second-strand cDNA, and finally the sense mRNA synthesis by T7 in vitro transcription. To evaluate the efficiency of SH, the presence of two overexpressed oncogenes associated with ESCC progression and metastasis (MAGE-A4 and CD44) was also analyzed in subtracted mRNAs. The results of RT-PCR and quantitative real-time PCR showed that high amount of MAGE-A4 and CD44 mRNAs were present in subtracted product. Furthermore, and in line with these results, no copy of housekeeping genes including GAPDH, β actin, and β2-microglobulinwas found in subtracted mRNAs. MAGE-A4 and CD44 gene expression were utilized to normalize the results of housekeeping gene expression analysis. Although establishing and optimizing of SH is difficult, our optimized stepwise process made it easy to use for rapid and specific isolation/amplification of tumor specific transcripts. This can assist better understanding of underlying molecular mechanisms involved in cancer development and introduce appropriate specific targets for cancer immunotherapy [48, 49].

Conclusion

This study presents subtractive hybridization in combination with SMART mRNA amplification as an efficient procedure to isolate tumor specific transcripts even with small amount of tumor tissue as primary RNA source. The isolated transcripts will be valuable candidate tumor markers for further analysis such as next generation sequencing to identify novel genes involved in ESCC progression and development. This approach can also utilize for other malignancies to detect novel transcript involved in cancer progression. Discovery of novel biomarkers in ESCC can improve prognosis, early diagnosis, as well as targeted therapy of cancer. The novelty of our SH technique is its coupling with mRNA amplification to increase of tumor-specific sequences as well as its unique and precise stepwise process. We applied this technique to successfully isolate differentially expressed transcripts which may lead to introduce crucial transcript involved in tumor progression and development, potentially utilizing in clinical immunotherapy of cancer.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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Informed Consent Informed consent was obtained from all individual participants included in the study.

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