Induction of HSF1 expression is associated with sporadic colorectal cancer

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Abstract

AIM: To explore the activation of signal transduction pathways related with the carcinogenesis of sporadic colon cancers.

METHODS: A gene array monitoring the activation of 8 signal transduction pathways (PathwayFinder GEArray) was used to screen the differentially expressed genes between colorectal cancer and normal colon tissues. The differentially expressed genes were further analyzed by RT-PCR, using RNA derived from colorectal cancer and normal colon tissue of 35 patients.

RESULTS: The expression of HSF1, HSF27, HSP90 and iNOS was increased in colon cancer tissues compared to normal colon tissue using PathwayFinder GEArray. The RT-PCR results showed that the expression of HSF1 was increased in 86% (30/35) patients and the expression of iNOS was increased in 63% (22/35) patients.

CONCLUSION: The induction of HSF1 gene expression is associated with sporadic colon cancer. HSF1 induces heat shock stress signaling pathway, which might play a role in the carcinogenesis of sporadic colorectal cancer.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancers in China. The incidence of CRC has increased over the last years and the fourth or fifth most prevalent cancers in China. The incidence of CRC increases 3.7% in Chinese cities annually(1) while the five-year survival rate has not improved significantly. Early detection and treatment remain a major factor for good survival rate of CRC. Therefore, exploration of early diagnostic markers and the mechanism study on CRC oncogenesis are in imminent need for early diagnostic and better treatments. Recently, mutation of APC tumor suppressor gene has been found to be responsible for initiating neoplastic process of familial adenomatous polyposis (FAP)(2). APC is a major regulator in the Wnt signal pathway that is activated due to APC mutation in FAP. The Wnt signal pathway is the oncogenic pathway for FAP. Although APC mutation was found in 60% sporadic CRC, Wnt signal pathway has not been proved to be an oncogenic pathway for sporadic CRC or any specific type of CRC. The oncogenic pathway for sporadic CRC remains to be determined.

Potten(3) has once summarized four signal pathways attributing to oncogenesis of hereditary CRC: APC-catenin-TCF-c-myc pathway (Wnt signal pathway) in FAP; microsatellite unstable pathway with mutation in hMLH1, hMSH2, hMSH6, hPMS1 or hPMS2 in HNPCC; p53 signal pathway with p53 mutation for CRC associated with chronic colon inflammation; estrogen receptor hypermethylated pathway for CRC associated with menopause. FAP and HNPCC are two hereditary CRCs. Other hereditary CRCs also includes Peutz-Jeghers syndrome and juvenile polyposis whose genetic predisposition is currently under investigation. The hereditary CRCs makes up less than 1/3 of CRCs while more than 2/3 of CRCs are sporadic. Therefore, it is important to identify oncogenetic pathways for sporadic CRC. As an open system, colon receives various chemical, physical and biological stimuli constantly. Some signals that vary among individuals according to their occupations, living environments and life styles. Some signal pathways that are activated by certain stimuli associated with particular occupations, environments and life styles may induce sporadic CRC. To explore the association between sporadic CRC and activation of signal pathways, we used PathwayFinder GEArray from SuperArray, a gene array capable of monitoring activation of 8 common signal transduction pathways to screen differential gene expression between colorectal cancer tissue and normal colon tissue. We found expressions of HSF1, HSF27, HSP90 and iNOS were increased in CRC tissue in comparison with normal colon tissue. RT-PCR was used for further confirmation. Our results suggest activation of heat shock stress signal pathway may attribute to oncogenesis of sporadic CRC.

MATERIALS AND METHODS

GEArray

PathwayFinder GEArray kit was obtained from SuperArray Bioscience Corp. (Frederick, MD, USA). It included reagents for probe generation and hybridization, and two identical gene arrays containing 23 marker genes for each array. The marker genes were used to monitor the activation of their associated pathways. The induction of marker genes suggested the activation of their associated pathways. The marker genes were transcriptional target genes of their associated pathways. These 8 signal transduction pathways and their associated marker genes are listed bellow.

Signal transduction pathways
Mitogenic signaling pathway
Stress signaling pathway
NFkB signaling pathway
NFAT signaling pathway
Anti-proliferation/TGFβ signaling pathway
P53 signaling pathway
CREB signaling pathway
Marker genes
egr-1, c-fos
c-myc, ATF-2, c-fos, p53, hsf1, hsp90, hsp27
iNOS, NFκB, ikB, c-myc
IL-2, FasL, CD5
p19, p21cα, p57kiki
p-myc
p21cα, pg7, pig8, mdm2, bax
cyp19, egr-1, c-fos
**Tissue and RNA preparation**
Specimens of colorectal cancer and normal colon tissues were obtained from 36 patients (age: 32-89 years) at the Second Affiliated Hospital of Zhejiang University. The membranes of cancer and normal tissues of each patient were separated and placed into liquid nitrogen immediately after the removal of cancer mass from the patients. Total RNA was prepared from each specimen using Trizol kit from GIBCO (San Diego, CA, USA) to derive total RNA from cancer tissue and normal tissue. One pair of specimens from a patient (patient 1) was used to perform gene array analysis and the rest of 35 pairs of specimens were used for RT-PCR analysis.

**Probe preparation**
Anneal Five µg total RNA was used to mix with 2 µL buffer A containing gene specific primers GEAprimers (SuperArray, Frederick, MD, USA) and water to bring up a volume to 20 µL.

Reverse transcription for probe labeling: A cocktail containing the following components was assembled in a 0.5 mL centrifuge tube containing 16 µL of 5x GEAlabeling buffer, buffer B (SuperArray, Frederick, MD, USA), 10 µL of [α-32P]-dCTP (370 GBq/L), 2 µL of RNAse inhibitor, 4 µL of MMLV reverse transcriptase (50 MU/L), 8 µL of RNAse-free H2O. The cocktail was placed in 42 °C water bath for 2 min followed by aliquoting 20 µL of the cocktail into the above primer annealed RNA from cancer tissue and 20 µL of the cocktail into above primer annealed RNA from normal tissue. After mixed, the mixture was placed in 42 °C water bath for 25 min for reverse transcription and labeling cDNA with α-32P.

Labeling termination: A 5 µLof 10x stop solution, buffer C (SuperArray, Frederick, MD, USA) was added to each reaction mixture to stop reverse transcription.

Probe denaturation: The labeled probe was placed in 94 °C heat block for 5 min, then immediately placed on ice until use.

**Hybridization**
Two PathwayFinder GEArrays were prehybridized with 10 mL GEAhyb solution (SuperArray, Frederick, MD, USA) for 2 h at 68 °C. The denatured probes obtained from cancer and normal tissues of patient 1 were then added separately to two 5 mL GEAhyb solution preheated to 68 °C. Each of the probe-containing GEAhyb solution was then used to hybridize with one PathwayFinder GEArray at 68 °C overnight. The GEArray was washed twice with 2×SSC, 10 g/L SDS for 20 min at 68 °C and twice with 0.1×SSC, 5 g/L SDS at 68 °C. X-ray films were placed on GEArrays to capture array images at -70 °C overnight.

**Density scan and analysis**
The hybridization signals on the X-ray film were scanned by IS1000 system to obtain digital number for its density. The average signal for each gene was calculated by the ratio of averaged value of β-actin or GAPDH.

**RT-PCR**
A 1 µg of total RNA obtained from each specimen was mixed with oligo (dT)12 for reverse transcription using MMLV (Promega, Madison, WI, USA) to derive the first strand cDNA. A pair of gene specific PCR primers was designed for HSF1, iNOS, MDR1 and β-actin genes. HSF1: CCATCCTGGGAGAGTAGTGTG AA for 5' end of the gene and GGCTCAGACGCCTGTCA CGA for 3' end of the gene. MDR1: GCCAAGCTTTAAGGGGCTATA for 5' end of the gene and GCTCAAGTAAAGGGGCTATA for 3’ end of the gene. β-actin: GCCA TCCTCACCCTGGAAGTA for 5’ end of the gene and GTCCAGACGCAGAGGATGGCA for 3’ end of the gene. PCR reaction was carried out at 95 °C for 3 min for denaturing followed multiple cycles at 95 °C for 15 s, at 56 °C for 10 s, at 72 °C for 45 s. For HSF1, iNOS and MDR1, the cycle number was 35 and for β-actin, the cycle number was 25. The PCR products were separated by electrophoresis and their quantity was determined by Kodak 1D image analysis software.

**RESULTS**
Identification of differential gene expression between cancer and normal colorectal tissues
Two identical PathwayFinder gene arrays from SuperArray (Frederick, MD, USA), each of which contained 23 marker genes monitoring the activation of 8 signal transduction pathways, were used to hybridize with labeled probes obtained from total RNA of cancer and normal colorectal tissues of a patient separately. The hybridized signal detected for each gene was normalized to the signal obtained for β-actin or GAPDH on the same gene array to derive gene expression value for each gene. After comparing gene expression value of each gene between two gene arrays, we found increased expressions of HSF1, HSF27, HSF90 genes of stress signaling pathway and iNOS gene of NFκB pathway in cancer tissue in comparison with normal tissue (Figure 1, Table 1).
activation of other NFκB pathways associated marker genes, the induction of iNOS expression might be due to the activation of other signal pathways other than NFκB signal pathways.

Table 1 Analysis of scanned density on gene arrays

| Gene | Ratio of cancer tissue vs normal tissue (β-actin) | Ratio of cancer tissue vs normal tissue (GAPDH) |
|------|-----------------------------------------------|-----------------------------------------------|
| Egr-1 | 1.16                                           | 0.94                                           |
| Hsf1  | 1.43                                           | 1.17                                           |
| iκB   | 1.23                                           | 1.00                                           |
| Mdm2  | 0.98                                           | 0.80                                           |
| Hsp27 | 1.58                                           | 1.28                                           |
| Hsp90 | 1.31                                           | 1.06                                           |
| iNOS  | 1.49                                           | 1.22                                           |

1 Ratio of cancer tissue versus normal tissue (β-actin): “Ratio to β-actin” from cancer/“Ratio to β-actin” from normal tissue; 2 Ratio of cancer tissue versus normal tissue (GAPDH): “Ratio to GAPDH” from cancer/“Ratio to GAPDH” from normal tissue.

Table 2 Comparison of gene expression between normal and cancer tissue by RT-PCR

| Patient | Dukes’ | HSF1 | iNOS | MDR1 |
|---------|--------|------|------|------|
| 1       | C      | 0.87 | 0.78 | 2.71 |
| 2       | B      | 2.13 | 2.57 | 0.64 |
| 15      | B      | 1.34 | 1.17 | 0.23 |
| 21      | A      | 1.68 | 0.81 | 1.71 |
| 29      | A      | 1.96 | 1.32 | 1.02 |
| 33      | C      | 1.71 | 0.72 | 0.64 |
| 34      | B      | 3.44 | 1.04 | 2.08 |
| 38      | B      | 1.78 | 1.17 | 0.87 |
| 40      | B      | 2.25 | 0.35 | 0.25 |
| 45      | B      | 2.07 | 0.44 | 1.50 |
| 48      | A      | 1.96 | 0.60 | 0.81 |
| 52      | B      | 1.75 | 1.83 | 0.35 |
| 71      | D      | 0.91 | 3.55 | 1.31 |
| 79      | C      | 1.11 | 0.39 | 0.99 |
| 82      | C      | 0.79 | 2.33 | 0.29 |
| 84      | C      | 1.53 | 1.07 | 1.04 |
| 85      | C      | 2.25 | 6.49 | 0.70 |
| 95      | C      | 1.64 | 0.88 | 0.40 |
| 99      | C      | 1.28 | 3.79 | 0.61 |
| 100     | A      | 1.70 | 0.62 | 0.16 |
| 104     | A      | 57.47| 2.77 | 0.47 |
| 105     | B      | 1.54 | 4.53 | 0.55 |
| 106     | C      | 4.52 | 4.97 | 0.25 |
| 113     | C      | 2.08 | 0.17 | 0.97 |
| 117     | C      | 1.84 | 2.49 | 0.87 |
| 118     | B      | 1.43 | 4.45 | 2.08 |
| 122     | C      | 1.78 | 0.57 | 0.25 |
| 125     | C      | 4.14 | 12.24| 1.73 |
| 128     | B      | 0.79 | 1.31 | 0.08 |
| 130     | C      | 1.07 | 0.73 | 0.73 |
| 131     | B      | 2.11 | 2.66 | 1.61 |
| 132     | C      | 0.73 | 1.55 | 1.20 |
| 142     | C      | 2.44 | 6.00 | 0.52 |
| 151     | B      | 2.16 | 1.06 | 4.31 |
| 153     | C      | 1.20 | 0.62 | 0.37 |

1 Ratio of HSF1, iNOS or MDR1 to β-actin; 2 [(HSF1, iNOS or MDR1)/β-actin, Cancer]/[(HSF1, iNOS or MDR1)/β-actin, Normal].

RT-PCR analysis for association of with sporadic colon cancer

A pair of gene specific PCR primers was designed for HSF1 and iNOS. RT-PCR was used to analyze the gene expression level of HSF1 and iNOS in both colon cancer and normal tissues of 35 patients. We confirmed increased HSF1 expression in 30 out of 35 patients in their cancer tissue in comparison to their normal tissue (Table 2). The average HSF1 expression value was 1.116±0.849 for normal tissue and 1.823±1.337 for cancer tissue. Using pair-wise t test analysis, the t value was 4.433 and P value was <0.001 (<0.05). Thus, it was statistically significant. The induction of HSF1 expression in tumor tissue was confirmed to be strongly associated with sporadic colorectal cancer. We also confirmed increased iNOS expression in 22 out of 35 patients in their cancer tissue in comparison to their normal tissue (Table 2). The average iNOS expression value was 0.867±0.761 for normal tissue and 1.133±0.843 for cancer tissue. Using pair-wise t test analysis, the t value was 1.803 and P value was 0.08 (>0.05). Thus, it was not statistically significant.

HSF1 was found to induce expression of MDR1. To explore whether HSF1 induction could increase expression of MDR1, we performed RT-PCR analysis on the same specimens from 35 patients. To our surprise, we found reduction of MDR1 expression in most (23/35) cancer tissues in comparison to normal tissues. The average MDR1 expression value was 1.099±0.885 for normal tissue and 1.443±1.096 for cancer tissue. Using pair-wise t test analysis, the t value was -2.329 and P value was 0.026 (<0.05). Thus, it was statistically significant.

Among sporadic CRCs from these 35 patients, 5 cases were at stage A, 12 at stage B, 17 at stage C and 1 at stage D. Five cases whose HSF1 expression did not increase in cancer tissue mainly belonged to late-stage patients (3 cases at stage C, 1 at stage D and 1 at stage B). However, statistical analysis could not be performed due to the limited number of cases.

DISCUSSION

Cellular signal transduction regulates almost all biological and physiological functions within cells. It is executed by a complex system where multiple signal transduction pathways interact together and are tightly controlled at different levels. The abnormality in cellular signal transduction can affect various cellular functions such as cell growth and proliferation, differentiation and metabolism and eventually lead to various diseases. Cancer is a typical disease resulting from abnormality in cellular signal transduction. Mutations of multiple genes in somatic cells are involved in the process of neoplastic formation. These mutations result in activation of oncogenes and inactivation of tumor suppressor genes, which lead to uncontrolled cell growth due to the loss of cellular differentiation and/or apoptosis ability. Further mutations in genes controlling cellular adherence and movement lead to tumor metastasis. Therefore, multiple signal transduction pathways in cell growth and proliferation, differentiation, apoptosis, adherence and movement are involved in initiation and progression of cancer.

There are four signal transduction pathways described by Potter[3,4] to be involved in the oncogenesis of hereditary CRC: APC-β-catenin-TCF-myc (Wnt) pathway[5], microsatellite unstable pathway[6], p53 pathway and estrogen receptor hypermethylation pathway. However, the signal transduction pathways involved in sporadic CRC remain unclear. APC mutation responsible for FAP’s oncogenesis was also found in 60-70% sporadic CRCs[7, 8]. Similarly, microsatellite instability was found in over 30% sporadic CRCs[9] and p53 mutation was found in 50% sporadic CRCs[10]. In addition, non-steroidal anti-inflammatory drugs (NSAIDs) were found to efficiently suppress tumorigenesis of CRC by inhibiting Cox-2 activity[11-14]. It indicates Cox-2 pathway is involved in CRC oncogenesis as well. These results suggest multiple signal pathways are involved in the oncogenesis of...
sporadic CRC. To efficiently identify signal pathways involved in sporadic CRC, we employed PathwayFinder gene arrays that utilize pathway target genes to monitor the activation of 8 important pathways related to cell growth, proliferation, survival, stress, apoptosis and inflammation. Monitoring induction of target genes by PT-PCR was found to be an effective way to identify the activation of Wnt signal pathway in adenomas of FAP compared to matched normal mucosa[15,16]. The utilization of gene array to identify induction of target genes greatly increased its efficiency.

We found HSF1 gene expression was significantly increased (30/35) in cancer tissue compared to its normal tissue in 35 patients. HSF1 stands for heat shock transcription factor 1 and is a transcription factor with a molecular weight of 82KD. HSF1 could be induced by heat, chemicals and hypoxia[17-19] and usually exists as a monomer in the cytoplasm. After heat stimulation, it became a trimer and could be translocated into nucleus[20]. Its transcriptional activity was greatly increased by the phosphorylation of serine induced by stimuli. HSF1 could activate transcription of its target genes by binding to a heat shock element[7]. The heat shock element was present in the promoter region of many genes such as HSP90, HSP27, HSP70 and drug resistant gene MDR[12,13]. Hoang[22] recently reported HSF1 expression was increased in a prostate cancer cell line. Our study not only provided the first evidence to demonstrate the association of HSF1 gene expression and CRC, but also suggested that the induction of HSF1 gene expression might be associated with a broad range of cancer. The reduced HSF1 expression in the remaining 5 cancer tissues might be due to different oncogenic mechanisms occurring in these CRCs from HSF1 induction related sporadic CRCs.

In this study, the induction of HSF1 gene expression was accompanied with the induction of HSP27 and HSP90 which were two target genes of HSF1, suggesting that the induction of HSF1 gene expression could activate HSF1 heat shock stress signal pathway in sporadic CRC. Heat shock stress signal pathway is highly involved in carcinogenesis since heat shock proteins (HSP90, HSP70, etc.) are responsible for maintaining the conformation, stability and function of key oncogenic client proteins involved in signal transduction pathways leading to proliferation, cell cycle progression and apoptosis, as well as other features of the malignant phenotype such as invasion, angiogenesis and metastasis[23-26]. HSP90 has currently served as an anti-cancer drug target for various drug development[27-29]. Also, HSP70 was found to inhibit apoptosis[30]. Our result suggested heat shock stress pathway contributed to carcinogenesis actively instead of passively as a bystander. Our hypothesis of heat shock stress pathway contributing to carcinogenesis of sporadic CRCs coincides with the fact that colon is an open system where it could retain and receive various kinds of “stress” substances for a long period of time. The “stress” substances might come from meat, alcohol, inflammatory tissue and others[31]. The prolonged exposure of these “stress” substances could lead to activation of heat shock stress pathway, thus leading to carcinogenesis through enhancing function of oncogenic proteins occurring in cells. The heat shock stress pathway could be further activated by hypoxia occurring in the tumor which was found to increase HSF1 expression as well[32,33]. Vegetables, nonsteroidal anti-inflammatory drugs (NSAIDs), hormone replacement therapy, and physical activity[34] could reverse the activation of heat shock stress pathway and therefore suppress carcinogenesis.

Hoang et al.[22] found HSF1 expression was increased in a prostate cancer cell line PC-3 derived PC-3M clone with metastatic ability compared to its parent cell line. By Western-blot and immunohistochemistry analysis of 18 prostate cancer and 4 normal prostate tissues, they confirmed the induction of HSF1 expression in prostate cancer tissues. However, since Western-blot and immunohistochemistry analyses detect protein expression instead of gene expression, it remains to be confirmed that the induction of HSF1 is at gene expression level. Our gene array analysis and RT-PCR directly measured mRNA level. We clearly demonstrated the induction of HSF1 was on the gene expression level and its strong association with sporadic CRCs. Nevertheless, in our study, each cancer tissue was paired with its matched normal tissue from the same patient for comparison of gene expression eliminating differential gene expression caused by individual genetic background.

PathwayFinder gene array from SuperArray provided two spots for each gene. The variation between the two spots was below 5%. According to Table 1, if β-actin expression level was used for normalization, genes whose expression was increased in cancer tissue included HSP27 (1.58 fold), iNOS (1.49 fold), HSF1 (1.43 fold), HSP90 (1.31 fold), ixB (1.25 fold), egr-1 (1.16 fold). However, if GAPDH expression level was used for normalization, genes whose expression was increased in cancer tissue only include HSP27 (1.28 fold), iNOS (1.22 fold), HSF1 (1.17 fold), HSP90 (1.06 fold). Therefore, it is important to use multiple housekeeping genes for normalization since expression of house keeping genes such as β-actin and GAPDH may fluctuate sometimes.

Vilaboa[31] found HSF1 could bind to the promoter region of MDR1 to increase its expression transcriptionally. However, MDR1 was also a target gene for Wnt signal pathway and the expression of MDR1 was increased in precancers lesion of FAP patients[32]. We found reduced MDR1 gene expression in most (23/35) cancer tissues. Our result suggested MDR1 gene expression was not regulated under heat shock stress signal pathway in colon tissue, more likely under Wnt signal pathway. The reverse association of MDR1 expression with HSF1 and β-catenin (Wnt pathway) indicated that different signal pathways contributed to the carcinogenesis of FAP and sporadic CRC. The reduced MDR1 gene expression was also found in other cancers such as kidney cancer[33] and brain cancer[34].

Recently, a large number of studies have revealed the association of iNOS expression with various cancers[35-39]. Yagihashi[40] studied 22 cases of colorectal cancer tissues by immunohistochemistry and RT-PCR and found increased iNOS expression was associated with colorectal cancer. Bing et al.[37] found increased expression of iNOS and production of prostanoids in colorectal cancer paralleled to the increase in COX-2, confirming the importance of this enzyme in colon cancer. Ropponen[39] found iNOS expression in colorectal cancer might be used as a prognosis marker as well. Our study also confirmed induced iNOS expression in 22 out of 35 cancer tissues, indicating that the biological significance of MDR1 and iNOS expression in CRC needs to be further studied.

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