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学位論文題目	Optimization of comparative expressed sequence hybridization for genome-wide expression profiling at chromosome level. (Comparative expressed sequence hybridization (CESH) による染色体レベルでの網羅的遺伝子発現解析の最適化)
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論 文 内 容 要 旨

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Background

Recent interest has been focused on which genes are up- or downregulated in diseases, whereas how the up- or downregulated genes are distributed on chromosomes remained largely unexplored. To this problem, many current researchers have adopted indirect, bioinformatics approach based on microarray data. Comparative expressed sequence hybridization (CESH) is recently developed alternative approach that enables direct global expression profiling at chromosome level. But there remain methodological problems in CESH..

Purposes

To improve the specificity and sensitivity of CESH and to demonstrate feasibility of CESH in global expression profiling.

Methods

- A gastric cancer cell line (KATO-III) and peripheral blood lymphocytes were used as test and reference samples, respectively.
- From DNA-free total RNA isolated from cultured cells, mRNA was selectively reverse transcribed, amplified and labeled in 3 ways. (1) transcription with oligo$(dT)_{24}$-T7 primer, amplification by T7 RNA transcription and cRNA labeling; (2) transcription with oligo$(dT)_{18}$ primer, DOP-PCR amplification and random priming labeling; (3) transcription with oligo$(dT)_{18}$ primer, DOP-PCR amplification and DOP-PCR labeling. Total RNA was also transcribed with random hexamer and then, amplified and labeled in 3 ways as in (2) and (3). As CESH analyses, these 5 kinds of probe sets (differentially labeled test and reference probes), non-amplified 6 probe sets (total RNA-derived and mRNA-derived cDNAs, each with 3 kinds of labeling), dye-reversal 5 probe sets, self-matched probe sets (differentially labeled same cDNAs) were hybridized to metaphase spreads. Fluorescent digital images were analyszed by PowerGene system (Applied Imaging). The fluorescence intensity ratio (T/R) of chromosomal regions represents the expression level of the test sample relative to that of the reference sample.
- Repeated purification by phenol/chloroform/isopropanol before and after labeling
- We also performed cDNA microarray analyses in triplicate using the probe set (1) and Human Cancer Chip (Version 4.0, TAKARA). To compare the microarray data with CESH profile, we calculated avarage T/R of array spots in 48 chromosomal regions, inferred the expected shifts of T/R and compare them with the actual T/R profile of CESH.
- The CESH results were compared with qRT-PCR data of 28 representative genes selected from the regions of differential expression detected by CESH.

(備考) 1．論文内容要旨は、研究の目的・方法・結果・考察・結論の順に記載し、２千字程度でタイプ等で印字すること。

2．※印の欄には記入しないこと。

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- Finally, we performed CESH with our optimized protocol between the cDNA with and that without demethylating 5-Aza-dC treatment to detect the methylated regions at chromosome level.

Results

1) In self-matched CESH, the T/R were evenly distributed around 1.0 in all the chromosomes except some centromeric regions in the cRNA labeling and the RP labeling, whereas, in the DOP-PCR labeling, significant shifts of T/R were seen in many chromosome arms.

2) Irrespective of the probe-labeling methods we used, the T/R ratio profiles of self-matched CESH were scarcely affected by the presence or absence of probe amplification either by T7-based RNA transcription or by DOP-PCR of cDNA.

3) Dye reversal CESH (RCESH) showed greater sensitivity than CESH.

4) Concordance of RCESH with cDNA microarray data was high in the sets of mRNA–derived probes with cRNA labeling or RP labeling, and low in conventional CESH or RCESH with total RNA-derived DOP-PCR-labeled probes.

5) Expression profile (of T/R) was very similar between cDNA microarray and qRT-PCR. Between qRT-PCR and RCESH with cRNA labeling or RP labeling, the expression levels were concordant except in 4 genes.

6) The optimized CESH between the cDNA with and that without 5-Aza-dC treatment disclosed 14 chromosomal regions that showed restoration of expression. Seven of these regions included genes that were reportedly methylated in Kato III.

Discussion

1) Our new protocol is summarized as: use of mRNA instead of total RNA, use of pre cDNA labeling or post-cDNA, RP labeling instead of DOP-PCR labeling, repeated purification before and after labeling and use of RCESH instead of CESH. From the practical point of view, RP labeling of cDNA may be preferable because of higher proportion of RNA-free processes. With this method, we have been successful in avoiding the false positive shifts encountered in conventional CESH even with metaphases of average quality. These findings suggest that the cause of false positive T/R shifts seen in the PCR labeling might be a labeling bias rather than the hybridization bias due to lower quality of metaphase.

2) CESH results were scarcely affected by probe amplification either by DOP-PCR or T7-based RNA transcription. Practically, DOP-PCR amplification may be preferable because higher amplification power of DOP-PCR than T7 transcription, enabling application to small samples, such as those microdissected from tissue sections.

3) Restoration of the expression after demethylating treatment was demonstrated by CESH at chromosome level. This supports the recently reported notion that epigenetic silencing can span large regions of the chromosome.

Conclusion

Our new protocol of CESH has been successful in avoiding false positive reactions of specified chromosomes in conventional CESH, and has enabled gene expression profiling in all the chromosomes.

別紙様式８（課程・論文博士共用）

学位論文審査の結果の要旨

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（学位論文審査の結果の要旨）

本研究は、染色体レベルで認識できるクラスターとしての遺伝子発現に注目し、それを解析するための CESH (comparative expressed sequence hybridization)の従来法の様々な技術的な問題点を克服し、信頼性の高い方法を確立したものである。この改良された方法を用いて、脱メチル化によって発現の亢進する遺伝子がクラスターとして存在し、プロモーターもメチル化も受けていることを明らかにし、クラスターとしての遺伝子発現が主として epigenetic に調節されていることを示唆した。また、染色体レベルの epigenetic な発現調節は、組織環境によって大いに変化しうることも明らかにした。

以上のように本研究は、CESH を大幅に改良し、クラスターとしての遺伝子発現解析における信頼性の高いツールとして利用するための道筋を切り開き、今後の研究に大いに貢献しうることを証明したものであり、本論文は、博士（医学）の学位論文に値するものと認められる。

（平成20年 2月 7 日）