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Stratification and delineation of gastric cancer signaling by in vitro transcription factor activity profiling and integrative genomics

Jayaprakash Periasamy [a], Muthulakshmi Muthuswami [a], Divya Bhaskar Rao [a], Patrick Tan [b], Kumaresan Ganesan [a,*]

[a] Cancer Genetics Laboratory, Department of Genetics, Centre for Excellence in Genomic Sciences, School of Biological Sciences, Madurai Kamaraj University, Madurai, India
[b] Duke–NUS Graduate Medical School Singapore, 8 College Road, Singapore

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ABSTRACT

Integrative functional genomic approaches are helpful in delineating the complex dysregulations in cancers. In the present study, in vitro activity profiling of 45 signaling pathway driven transcription factors in eight gastric cancer cell lines and direct comparison with genome-wide profiles of gastric tumors were performed and the integration resulted in the identification of three categories of factors/pathways: i) highly activated signaling pathways that stem from mutations are the critical oncogenic drivers, ii) constitutively activated stress responsive pathways which are activated not due to genetic alterations, and iii) consistently down-regulated nuclear receptor responsive factors. This functional profiling helps in discriminating therapeutic targets and signaling interactions.

1. Introduction

Several successful therapeutic targets have been established in targeted cancer therapeutics [1]. Due to the complex and heterogeneous nature of cancers as well as due to drug resistance, additional and improved therapeutic targets and drugs need to be constantly identified [2–7]. In the process of carcinogenesis, the acquired defects in genes are selected to the survival of cancer cells and progression. Multiple gene defects culminate to a specific biological process and complex network of interactions among genes is involved in executing a biological or cellular functional process [8]. Complexity and the driving factors of cancers are routinely analyzed from somatic mutations, gene copy number alterations, chromatin and epigenetic codes, gene transcription, protein quantity, and protein functionality [9–13]. These genetic alterations and the resultant deregulations are contributing in the development and progression of cancer. Therefore, investigation of these alterations and deregulations is a fundamental method in understanding the processes of carcinogenesis, clinical cancer diagnosis and therapeutic targeting [14–17]. Modern unbiased genomic approaches have tremendously contributed to the understanding of the complex genetics of cancers against the conventional dependence on candidate gene based approaches [18–22]. With the recent advancements in genomics, new cancer genes are being identified and it further increases the complexity in choosing therapeutic targets [23].

Growth regulatory signaling pathways are driven by multiple genes and the aberrations in signaling pathway component genes would result in aberrant growth and proliferation of cancer cells [24–29]. Functional assays which measure the collective functionality of multiple genes are more important in understanding the driving forces in cancer cells. Cellular signaling pathways are modulated owing to the activation or repression of several genes and proteins [30]. Signaling pathways are the central regulators of cell fate and respond to multiple external and internal stimuli. Deregulation in signal transduction is increasingly observed in cancers and became impressive and realistic targets in cancer therapeutics [31]. Most of the oncogenes and tumor suppressor genes are component of one to few signaling pathways [32–36]. For these reasons, rather than genes and proteins, signaling pathways are considered as realistic and measurable therapeutic targets. Signaling pathway deregulations are hallmarks of cancer cells and hence routinely quantified in cancer biological investigations by analyzing target gene expression, protein expression, kinase activities, pathway gene mutations and transcription factor activation by reverse phase protein arrays, suspension arrays, reporter assays and tandem arrays [37–44]. Reporter assays measure the in vitro transcriptional activity of the transcription factors which are involved in genomic signaling by the transcriptional activation of target genes [45]. Apart from measuring the cellular signaling activity, reporter assays also are indicative of the functionality of a set of genes (few to hundred) and thus being extensively meaningful measures in functional genomics. Selected candidate pathways are routinely investigated by transcription factor activity based reporter assays. Very

Abbreviations: TRE, transcription factor response element; CNV, copy number variations; GEO, gene expression omnibus; ER stress, endoplasmic reticulum stress.

* Corresponding author at: Department of Genetics, School of Biological Sciences, Madurai Kamaraj University, Madurai 625021, India.
E-mail address: kumar@oncocellomics.org (K. Ganesan).

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few studies have attempted to analyze a larger panel of transcription factors in a panel of cancer cell lines [44,46,47]. In this study, we analyzed the signaling pathway activities by measuring the pathway driven transcription factor activity. We used a gene reporter array approach to quantitatively analyze the transcriptional activities of transcription factors regulated by various signaling pathways for distinct biological processes like growth regulation, immune response and stress response. In total, we profiled 45 different transcription factor activities across 8 gastric cancer cell lines and this approach seems useful in discriminating the potential cancer therapeutic targets from bystanders in cancer cells. Apart from recapitulating the known concepts in cancer biology, we also identified the novel interactions between oncogenic and stress signaling pathways from this comprehensive profiling and integrative functional genomic analysis.

2. Materials and methods

2.1. Cell culture

The human gastric adenocarcinoma cell line AGS was obtained from National Centre for Cell Science (NCCS), Pune, India. Kato-III was obtained from ATCC, USA, and MKN45 cells were from Japan Health Science Research Resource Bank. Gastric cancer cell lines YCC1, YCC3, YCC11 and YCC16 were a kind gift of Dr. Sun Young Rha, Yonsei Cancer Centre, Korea. AGS cell line was cultured in Dulbecco's Modified Eagle Medium/Nutrient mixture F-12 Ham (HiMedia, India) supplemented with 10% fetal bovine serum (Sigma), Kato-III was cultured in RPMI-1460 (HiMedia, India) supplemented with 20% FBS, MKN45 cells in RPMI-1640 with 10% FBS and YCC1, YCC3, YCC11, and YCC16 cell lines were maintained in MEM (HiMedia, India) with 10% FBS. Along with FBS, the media was supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 1.5% sodium bicarbonate and 2 mM L-glutamine.

2.2. Chemicals and reagents

45 signaling pathway reporter plasmid panels (CCA-901L) were from SA Biosciences. pGL3-Basic Vector and pGL3-Control vector were from Promega. siRNA for targeting *CTNNB1*, *POU5F1* (*OCT4*), *IRF1*, *RELA*, *HIF1A*, *YY1* and *NFYA* were from Dharmacon (siGENOME). FuGENE HD transfection reagent from Promega was used for plasmid transfection. Oligofectamine reagent for siRNA transfection was from invitrogen. D-Luciferin (Life Science Technologies) and Coelenterazine (Gold Biotechnology) were used for the preparation of dual luciferase assay reagents.

2.3. Pathway profiling in gastric cancer cell lines by 45 pathway reporter arrays

Gastric cancer cell lines were seeded in 96 well cell culture plates and transfected with 45 signaling pathway reporter array panels of plasmids (SA Biosciences). The plasmids used for 45 signaling pathways are: pSTAT3-FLUC, pIRF1-FLUC, pRXR-FLUC, pWNT-FLUC, pE2F1/DP1-FLUC, pMYC/MAX-FLUC, pATF4/3/2-FLUC, pAR-FLUC, pNRF2/1-FLUC, pATF6-FLUC, pC/EBP-FLUC, pCREB-FLUC, pE2F/DP1-FLUC, pP53-FLUC, pEGR1-FLUC, pCBF/NF-Y/YY1-FLUC, pER-FLUC, pGATA-FLUC, pGR-FLUC, pHSF-FLUC, pHNF4-FLUC, pMTF1-FLUC, pGLI-FLUC, pHIF-1-FLUC, pSTAT1/STAT2-FLUC, pSTAT1/STAT1-FLUC, pKLF4-FLUC, pLXRa-FLUC, pElk-1/SRF-FLUC, pAP-1-FLUC, pMEF2-FLUC, pNANOG-FLUC, pNFκB-FLUC, pRBP-Jk-FLUC, pOCT4-FLUC, pPAX6-FLUC, pFOXO-FLUC, pNFAT-FLUC, pPPAR-FLUC, pPR-FLUC, pRAR-FLUC, pSOX2-FLUC, pSP1-FLUC, pSMAD2/3/4-FLUC, pVDR-FLUC, AHR-FLUC, CMV-FLUC and CMV-Renilla. In each well, a pathway specific reporter plasmid (Fluc) was transfected along with renilla luciferase internal control plasmid (CMV-RLUC) at 100:1 ratio. 48 h after transfection, cells were harvested and then dual luciferase assays were performed. For luciferase reporter assays, the cells were lysed with 1× passive lysis buffer (Promega) and the luciferase activity assays were performed by dual-luciferase reporter assay protocol [48]. Firefly and Renilla luciferase activities were measured in Spectramax L microplate luminometer (Molecular Devices). All the experiments were performed in duplicates for independent times.

2.4. Antibodies and western blotting

Total proteins extracted from gastric cancer cell lines were used for the analysis of multiple signaling proteins. Specific antibodies were used for the detection of cellular levels of β-catenin (1:1000; Calbiochem, 219353), RXRα (1:200; Santa Cruz, sc-553), Grp78 (1:200; Santa Cruz, sc-1050), NFkB p65 (1:200; Santa Cruz, sc-7151), p53 (1:200; Santa Cruz, sc-126), β-actin (1:5000; Sigma-Aldrich, A1978), HIF-1α (1:200; Santa Cruz, sc-13515), and Nrf2 (1:200; Santa Cruz, sc-722) with the specified antibodies and the dilutions specified in the parenthesis. The secondary antibodies used were anti-mouse IgG-HRP (1:5000; GE Healthcare, NA9310), anti-rabbit IgG-HRP (1:5000; GE Healthcare, NA9340), and anti-goat IgG-HRP (1:5000; A5420, Sigma Aldrich). Blots were developed using ECL Prime reagent from GE Healthcare.

2.5. Staining of endogenous reactive oxygen species (ROS)

To stain endogenous ROS, gastric cancer cell lines grown in normal growth media were changed to complete growth media without phenol red. Upon 12 h of incubation, the cells were harvested and stained with H_2DCFDA (Invitrogen) for 45 min at CO_2 incubator in the dark. Subsequently, the cells were stained with propidium iodide (PI) for the discrimination of dead cells. Stained cells were analyzed through FITC and PI channels in flow cytometer FACS Aria III (BD Biosciences), and FITC(+)/PI(−) cells were plotted as ROS positive population.

2.6. Analysis of genetic alterations in gastric cancer cell lines

Analysis of somatic mutations and copy number alteration in gastric cancer cell lines was performed by retrieving exome sequencing and copy number profile data from Cancer Cell Line Encyclopedia (CCLE) through cBioPortal for Cancer Genomics (http://www.cbioportal.org/public-portal/study.do?cancer_study_id=ccle_broad) [49,50]. Genes mutated in 38 gastric cancer cell lines were checked for pathway alterations by DAVID gene-set enrichment tool [51]. Gene mutation, amplification and deletion information were integrated with in vitro pathway reporter data to infer pathway deregulation by alterations in the component genes (Fig. 4A, B). For the analysis of genetic alterations, independent copy number variation (CNV) and Kinome sequencing information from Zang et al. [52] were used (*CTNNB1* amplification, *CDH1* deletion and *APC* deletion information in Fig. 4A, *TP53* mutation & deletion in Fig. 4B and mutation, amplification, deletion information for MAPK genes shown in Fig. 4C).

2.7. Selection of consensus differentially expressed genes and transcription factor enrichment analysis

Microarray gene expression profiles used in the study were downloaded from Gene Expression Omnibus. The normalized expression profiles of four datasets (GSE13911, GSE2685, GSE17154 and GSE19826) comprising a total of 82 tumors and 93 normal gastric tissues were analyzed independently to identify the differentially regulated genes. Two tailed t-test with p-value <0.05 and fold change >1.5 (up-regulation) or <-1.5 (down-regulation) were used as criteria for filtering the differentially regulated genes in gastric tumors when compared to normals. A vote counting based method [53] was used in which occurrence of a gene in each independent profile was analyzed and filtered the genes that are commonly up/down-regulated with minimum significant fold difference of 1.5 in at least 3 out of the 4 profiles. Thus derived 217 up-regulated and 253 down-regulated genes were used for further