Review Article

Next generation sequencing-based emerging trends in molecular biology of gastric cancer

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Abstract: Gastric cancer (GC) is one of the leading causes of cancer related mortality in the world. Being asymptomatic in nature till advanced stage, diagnosis of gastric cancer becomes difficult in early stages of the disease. The onset and progression of gastric cancer has been attributed to multiple factors including genetic alterations, epigenetic modifications, *Helicobacter pylori* and Epstein-Barr Virus (EBV) infection, and dietary habits. Next Generation Sequencing (NGS) based approaches viz. Whole Genome Sequencing (WGS), Whole Exome Sequencing (WES), RNA-Seq, and targeted sequencing have expanded the knowledge base of molecular pathogenesis of gastric cancer. In this review, we highlight recent NGS-based advances covering various genetic alterations (Microsatellite Instability, Single Nucleotide Variations, and Copy Number Variations), epigenetic changes (DNA methylation, histone modification, microRNAs) and differential gene expression during gastric tumorigenesis. We also briefly discuss the current and future potential biomarkers, drugs and therapeutic approaches available for the management of gastric cancer.

Keywords: Gastric cancer, next generation sequencing (NGS), microsatellite instability (MSI), single nucleotide variations (SNVs), epigenetic modifications, differential gene expression

Introduction

Gastric Cancer (GC), accounting for 8.8% of all cancer related deaths, remains the third most common cause of cancer related mortalities worldwide. GC is more prevalent in males (67.3%) in comparison to females (32.7%) [1]. Prevalence of GC also shows a demographic variation with approximately half of the global occurrence confined to East Asian countries. GC incidence rate shows a drastic difference between China and USA with 46.5 and 8 GC cases per 1,00,000 people, respectively. This data implies a possibility of association of gender and ethnicity with the occurrence of GC. Early diagnosis of the disease is difficult as manifestation of symptoms takes a substantial period of time. Lauren [2] categorized GC into intestinal type and diffuse type, the former being more prevalent in high-risk areas and the other type in low risk areas [3]. WHO has classified GC on the basis of its histological patterns into tubular adenocarcinoma, papillary adenocarcinoma, mucinous adenocarcinoma, poorly cohesive carcinomas and mixed carcinoma. A number of risk factors have been found associated with the occurrence of GC, including infection with *Helicobacter pylori* and Epstein-Barr Virus (EBV), dietary habits, smoking, consumption of alcohol and red meat [4]. In addition to these risk factors, existence of genetic susceptibility has been emphasized and defined as a major cause of gastric tumorigenesis on the basis of mutations in different genetic elements and epigenetic modifications. Recent advancements on these lines have motivated researchers to take up comprehensive genetic and genomic analysis of gastric tumorigenesis. Application of Next Generation Sequencing (NGS) technologies exploiting whole genome sequencing to targeted sequencing has played an important role in the identification of the genetic variations and anomalies leading to the development of GC. NGS, not only provides a high through-put, cost effective and faster technology, but also offers a more comprehensive and accurate tool for genome analysis [5, 6]. The edge of NGS over Sanger’s method in sensitivity and depth is evident by the fact that percentage detection of allele frequency in NGS is 2-10% as compared to 15-25% in Sanger’s sequencing [7]. Owing to these advantages,
NGS has profoundly been applied in the field of cancer biology for identifying genetic aberrations underlying tumorigenesis.

All the four NGS-based approaches i.e. Whole Genome Sequencing (WGS) [8], Whole Exome Sequencing (WES) [9], RNA Sequencing (RNA-Seq) [10] and targeted sequencing [11] have been exploited for the detection of the genetic and epigenetic changes implicated in GC. As the terminology suggests, WGS represents sequencing of the complete genome facilitating detection of SNPs, InDels, copy number changes and large structural variants in the target genomes. Unlike WGS, WES deals with the sequencing of only exons or coding regions of the genome, which although representing less than 2% of the genome, contains ~85% of the known disease-related variants [12]. Disparate to WGS and WES, RNA-Seq identifies changes in the transcriptome.

This approach has been useful in the detection of alternative gene-spliced transcripts, post-transcriptional modifications, gene fusions, single-nucleotide polymorphisms (SNPs), and changes in the level of gene expression. Next, targeted sequencing is an economical and time saving technique, when a set of specific genes need to be explored. It includes sequencing of exome, specific genes of interest (custom content), targets within genes, or mitochondrial DNA. A summary of important NGS-based studies in GC is presented in Table 1. The Cancer Genome Atlas (TCGA) categorises MSI+ GC (22%) as a subset of GCs along with EBV+ (9%), chromosomal instability (CIN; 50%) and genomically stable (20%) GC [13] (Figure 1). Different factors contributing towards the onset and progression of GC are enumerated in Figure 2.

In this review, we summarize the application of NGS technology in determining genetic and epigenetic modifications along with differential gene expression implicated in the molecular pathogenesis of gastric cancer. We also provide useful information about drugs developed or under clinical trials for the treatment of GC and their possible target sites.

### Table 1. Summary of NGS approaches applied to study molecular biology of gastric cancer

<table>
<thead>
<tr>
<th>Sequencing approach</th>
<th>Sample source</th>
<th>Platform</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
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<td>[35]</td>
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<td>[30]</td>
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<td>Illumina</td>
<td>[40]</td>
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<td>Tissue/Blood</td>
<td>Ion Torrent</td>
<td>[34]</td>
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</table>

Figure 1. TCGA classification of different subtypes of gastric cancer.
Molecular biology of gastric cancer

Genetic alterations in gastric cancer

Variation in microsatellite sequences: Microsatellites, also known as Simple Sequence Repeats (SSRs), refer to 1-6 base long tandem DNA repeats in the genome. These repeats have been found to be hypervariable incorporating insertions and deletions arising during replication and recombination events with mutation rate being $10^{-4}$ times higher in comparison to that at non-repetitive loci. Such expansion and contraction changes in the microsatellite regions have commonly been termed as microsatellite instability (MSI). Implication of microsatellite instability has been explored widely in many cancers, especially colorectal cancer, gastric cancer, ovarian cancer, and head and neck cancer. Microsatellite unstable tumors can be graded into two distinct MSI phenotypes: MSI-high (MSI-H) and MSI-low (MSI-L). MSI and related changes have been implicated in about 15-30% cases of gastric cancer [14, 15].

MSI influences cancer development by modulating the expression pattern of many mismatch repair (MMR) genes, tumor suppressor (TS) genes and oncogenes. While tumor suppressor genes and oncogenes work by controlling cell proliferation, apoptosis, immune evasion and angiogenesis in carcinoma, mismatch repair genes are responsible for correction of the base-base mismatches and insertion or deletion impair repair caused during DNA replication and recombination events. Genetic instability at microsatellite loci in MMR genes caused by different processes including DNA polymerase slippage and unequal crossing over leads to the production of truncated or altered products of these genes. Such aberrations in mismatch repair system are responsible for cell’s inability to correct replication errors in downstream target genes, thereby affecting their normal expression. Figure 3 explains the outcome of different molecular events causing instability in a microsatellite sequence.

Genetic and epigenetic modifications in DNA mismatch repair (MMR) genes result in a mutator phenotype. MSI mainly accumulates frame-shift mutations at microsatellite loci located in the coding regions of a target tumor suppressor or other tumor-related genes [16-18]. MSI+ GCs show epigenetic alterations such as hypermethylation of various genes including the key MMR gene MLH1. The differences in genotype and phenotype between MSI+ and MSI- GC are likely linked to other differences in biological and clinical features. Recent findings from NGS analysis such as the frequent mutation of the AT-rich interactive domain 1A (ARID1A) in MSI+ GCs support this notion [19].

WGS and RNA-Seq analyses of GC samples from Korea have revealed a total of 18,377 mutations at different microsatellite loci with five or more repeat units in coding and untranslated regions, suggesting a role of microsatellite sequences in protein synthesis and carcinogenesis [8]. Further, deletion mutations were identified at 14,895 MS loci, of which 3,482 were detected exclusively through RNA-Seq. Using Selective Target database (SeiTargbase), 24 candidate genes having deletion in their CDS were selected on the basis of driver gene score and pathway analysis, and subsequently
NGS based molecular biology of gastric cancer

Figure 3. Different molecular events and their consequences leading to microsatellite instability shown in a hypothetical microsatellite sequence.

validated through Sanger sequencing. Mutations within mononucleotide tracts in TGFBR2, CEP164, MIS18BP1, RNPC3, KIAA2018, CNOT1 and CCDC150 genes were detected in
more than 63% of the MSI-H GC. Low to indiscernible gene expression was detected when frame shift mutations were located in CDS (23 genes), 5’UTR (13 genes) and 3’UTR (186 genes). A comparative analysis of UTR mutated genes revealed lower expression levels for UTR MSI genes in comparison to those lacking these mutations. Deletion at (A)n repeats in the coding region of TGFBR2 gene caused a loss of expression in MSI-H samples [8].

A high mutation rate in chromatin remodelling gene ARID1A has been found associated with instability at microsatellite loci. Mutations in ARID1A gene have also been suggested to be linked with concurrent mutations in PIK3CA gene. An analysis of high frequency of PIK3CA mutations in MSI+ gastric cancers has revealed the potential of PIK3CA inhibitors in the personalized treatment of MSI+ patients [20]. Moreover, ARID1A displayed a gamut of protein inactivating mutations in different molecular subtypes of GC (83% MSI+ GC, 73% EBV+ GC and 11% MSS, EBV- GC). In the MSI GC samples, 97% of the mutations were InDels, mostly involving mononucleotide repeats of C or G (89%). A G7 tract located in exon 20 of ARID1A was found mutated in 26% of MSI+ gastric cancers. For the MSS gastric cancer samples (both EBV infected and non-EBV infected), 59% of the mutations were SNVs with 6 nonsense and 4 missense mutations. Of these, only seven mutations were InDels, with one involving a mononucleotide repeat sequence. ARID1A gene contains many short repeats of 4-7 mononucleotides in its coding region. The overall mutation rate of ARID1A in MS instable GC (78%) is comparable to that of well-established and functionally validated driver genes inactivated by MSI, such as TGFBR2 [21]. Absence of ARID1A alterations is an independent predictor for early recurrence of GC while ARID1A alterations (mutation or protein deficiency) were related to longer progression-free survival (PFS) of GC patients. Wang et al. (2011) provided an explanation that ARID1A alterations might be a characteristic of a special GC subgroup, driven by epigenetic factors.

Exome sequencing of 22 GC patients revealed an average of 31.61 somatic mutations including both SNVs and InDels per megabase of DNA in MSI+ GC samples in comparison to 3.29 in the MSS GC samples recording an approximate tenfold change, expected as aftermath of a defective mismatch repair system. MSI+ GC samples had a markedly higher frequency of T to C transitions (30%) and tenfold higher number of protein-altering somatic mutations in comparison to MSS GC samples [21]. The reported somatic mutation rate in MSS GC samples was higher at 1.19 per Mb in comparison to that reported in earlier studies [22].

Good prognosis of cancer is characterized by a hypermutated profile showing at least one mutation in 90.5% cases comparative to the poor prognosis subgroup with at least one mutation in 46.2% cases. The median mutation rate (total number of mutations/total number of cases) in the good prognosis group remained 2.0 per sample, whereas in the poor prognosis group this figure being 0.9 per sample. Moreover, the good prognosis subgroup showed MSI in 42.9% cases compared to 7.7% in the poor prognosis subgroup [23].

A remarkable association of PIK3CA mutations with MSI phenotype was observed in GC. Pyrosequencing of MSI cancer samples revealed mutations in exon 1, exon 9 and exon 20 of PIK3CA and their frequency was significantly correlated with the level of MSI [24]. MSI in coding regions has other functional consequences also including lower average transcript levels. MSI frequency is also associated with chromatin organization and nucleosome positioning [18]. Another study [21] reported significantly higher frequency of protein altering mutation in MSI tumors compared to that in MSS samples. In MSI samples, 16 significantly mutated genes including known oncogenes, KRAS and ERBB2, were identified. Other potential novel driver candidates are ZBTB1, TRAPPC2L, as well as G protein-coupled receptors GPR39, GPR85 and CHRM3 [21].

A comparative whole genome analysis of microsatellite and chromosome unstable GC patients by Nagarajan and colleagues [25] in 2012 found 14,856 somatic SNVs (11,738 InDels) in microsatellite unstable sample and 17,473 somatic SNVs (2,486 InDels) in chromosomal unstable sample with an average mutation frequency of five per Mb of the genome. More than 100 SNVs were discovered to be located in the protein coding regions for each tumor type [25]. Exome specific somatic variants (5,588 SNVs and 2,347 InDels) were identified with a five times higher frequency through exome sequ-
### Table 2. Type and frequency of mutations implicated in gastric cancer

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>Target</th>
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<td>SNV (93.7) Insertion (6.2)</td>
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<td>ARID1A</td>
<td>SNV (45.4) Deletion (36.36) Insertion (18.18)</td>
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<td>SNV (88.88) Insertion (11.11)</td>
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<td>SNV (100)</td>
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<td>SNV (75) Deletion (25)</td>
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encing of 37 GC samples comparative to that in other contemporary sequencing studies [20, 21], highlighting the statistical advantage of whole-genome analysis for studying mutation signatures in gastric tumorigenesis. The MSI+ tumor exhibited an excess of SNVs in protein coding regions and a striking seven-fold higher frequency of micro-indels but lack of large-scale SNVs and amplifications or deletions. In MSI+ GCs, ACVR2A, RPL22, LMAN1, and STA2 showed recurrent single base thymine deletions in poly (T) regions, later confirmed through screening of additional 94 gastric cancer/normal paired samples. Mutations in ACVR2A, RPL22 and STA2 at (T)\(_n\) MS locus were observed in 86%, 64% and 29% of MSI+ GC tumors, respectively. Mutations in LMAN1 at (T)\(_n\) MS locus were present in 50%, of MSI+ GC tumors. ACVR2A, a gene found to be recurrently mutated in MSI+ colorectal cancer [26] was also observed in MSI+ GC also [25] indicating the probability of existence of common key players between the two types of cancers. Also, the frequency of mutations seen here was comparable to the previously reported frequency in MSI+ colorectal cancers [27, 28] emphasizing the importance of ACVR2A and TGF-β signalling in MSI+ GC. The oncogenic role of RPL22 and LMAN1 requires further investigations [25].

The foregoing discussion clearly suggest that NGS has proved to be an advancement over the traditional Sanger’s sequencing in delving different features of MSI related factors implicated in gastric tumorigenesis. Instead of relying on forward and reverse reads of microsatellite bearing gene(s), availability of millions of NGS reads of hundreds of microsatellite containing genes allow high throughput search for MSI alterations with more accuracy generating huge amount of reliable low cost data with amazing speed.

**Single nucleotide variations, InDels and copy number variations**

Genetic aberrations like insertions, deletions, SNVs and SNPs are mutations that vary from a single base pair change to a few base pair change in a region of the genome. Both SNV (Single Nucleotide Variation) and SNP (Single Nucleotide Polymorphism) are single base pair substitutions with different frequency of occurrence in a population. Recent advancements in NGS techniques have proved their importance in revealing individual specific variations instead of common mutations across genomes routinely done through earlier sequence analysis techniques. Table 2 summarizes data on various single nucleotide mutations associated with gastric cancer.

Kuboki et al. [29] analysed 409 cancer related genes in 121 advanced stage GC samples to detect copy number variations and mutations using targeted NGS. The top mutated genes showing 8-36% mutation frequency were TP53, SYNE1, CSMD3, LRPIB, CDH1, PIK3CA, ARID1A and PKHD. The relative reading depth to the reference (RRDR) of an individual gene was calculated for the analysis of copy number variation keeping RRDR of >2 as indicator of copy number variation in the study. Out of the 409 genes studied, 203 genes showed RRDR values of >2 and the percentage of samples with CNV ranged 0.8-20% [29].

Gain in DNA copy number with high mRNA level through Illumina microarray has been analysed in 50 gastric adenocarcinoma samples. Majority of the genes with increased level of mRNA were present on chromosomal regions 20q and 8q indicating that amplifications at these locations have greater effect on mRNA level. There is concurrence in data on mutations obtained by deep sequencing and genotyping arrays. Out of 18,549 mutations, 3,357 somatic variants were nonsynonymous and exonic. The observed alterations were located in genetic elements participating in different pathways like WNT, Hedgehog, cell cycle, DNA damage and epithelial-to-mesenchymal-transition pathway. A nonsense germline mutation (c.1023T>G) in CDH1 gene causing premature formation of stop codon resulting in low level of transcription has been described in different studies [30, 31]. Another mutation in CDH1 gene (c.1849G>A) detected in GC has also been reported in other cancers like endometrial and breast cancer [32, 33].

TCGA has categorised significantly mutated genes into two panels to assess the utility of panel based targeted sequencing. Twenty genes were placed in one group (selective hotspot panel) while 58 genes were included in the other group (comprehensive panel) in 21 resected GC specimens. TP53, MUC6, APC and SYNE1 genes were among the most mutated genes in patients with early stage of GC [34].
Copy number variation (CNV) has been detected for KRAS, JAK2, CD274 and PDCD1LG2 genes applying three whole genome amplification methods of single cell resequencing [35]. A total of 27,732 somatic mutations were identified using exome sequencing, out of which 40% were protein altering (8,726 missense, 1,661 InDels, 494 nonsense, 10 stop loss and 221 essential splice site) mutations. The altered pathways included TP53, RTK, PI3K and cell cycle pathway. ERBB2 point mutations in GC were found to be different from the activating point mutations in breast cancer [36, 37].

RNA-Seq data showed an inframe deletion of 26 residues which disrupts the domain essential for protein kinase activity, thereby losing the tumor suppressing potential of MAP2K4 [37]. Zang and colleagues [38] have characterized the protein coding regions of 537 kinases in 14 commonly studied cell lines using NGS and detected more than 300 novel kinase SNVs. A family wise analysis further revealed a significant SNV enrichment in MAPK related genes.

Recurrent point mutations in various genes including TP53, PIK3CA, CDH1, KRAS, RHOA, ERBB2, ERBB4, were analysed in regular GC while TP53, PIK3CA and KRAS were also found to be significantly mutated in hypermutated GC. CDH1 and SMAD4 mutations were significantly associated with shortened survival of GC patients [39]. Mutations were detected in prognostically selected (good prognosis and bad prognosis) groups in GC patients revealing that PIK3CA, KRAS and TP53 represent the highly mutated genes in the good prognosis group. The poor prognosis group showed a lower mutation rate in comparison to that observed in the good prognosis group. High frequency of mutations in TP53 gene was reported in 25 archival gastrointestinal samples using Illumina MiSeq platform [40]. A total of 737 targets in 45 genes representing oncogenes and tumor suppressor genes were analysed in 238 GC samples revealing missense point mutations in TP53 in 9.7% population [41]. Moreover, 58% mutation in KIT and 26% mutation in PDGFRA were also reported [42].

Using targeted multigene sequencing, 46 cancer related genes were explored in five GC samples, out of which TP53 and PIK3CA were found mutated in 60% and 40% samples, respective-ly [43]. A study reported whole genome sequencing of 30 diffuse type GC samples and observed recurrent RHOA mutations which were confirmed through further validation experiments. Mutations were observed in RHOA in 22 out of 87 cases [44].

**Epigenetic modifications**

One of the crucial mechanisms that steer the onset of cancer is the occurrence of widespread epigenetic modifications that can lead to abnormal gene expression and genomic instability. NGS technologies have surpassed array techniques applied in earlier methylation studies by providing high density coverage of the epigenome. Methylation across the genome is unravelled through whole genome bisulfite sequencing as well as targeted sequencing aiming screening of the specific desirable regions of interest.

An epigenetic trait has been defined as a “stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence” [45]. Any abnormality in the epigenetic system has been attributed as pathogenic mechanism causing the initiation and progression of several complex diseases. A vast amount of research has been conducted linking aberrant DNA methylation profiles and histone modifications to developmental defects, obesity, asthma, cancers and neurodegenerative disorders [46]. However, given the complexity of epigenetic mechanisms, which are influenced by aging, genetic variations, such as polymorphisms, and environmental factors, there is still a long way towards collecting, researching, and deciphering epigenetic information [47, 48]. Translation of all these mechanisms into relevant biological information requires an integrated approach of research covering related fields. These epigenetic alterations either accelerate or decelerate the cell’s transcription machinery thereby regulating the expression of genes in the concerned section of chromatin [49-51]. Epigenetic changes are somewhat similar to genetic mutations that change the underlying structure of the DNA, contributing towards the initiation and progression of cancer [52]. For normal gene expression, epigenetic machinery responsible for DNA methylation, DNA hydroxymethylation, post-translational modifications (PTMs) of histone proteins, nucleosome remodelling, and regulation
Aberrant DNA methylation in the promoter region of genes that leads to inactivation of tumor suppressor and other cancer-related genes is the most well-defined epigenetic hallmark in GC. In mammalian cells, DNA methylation consists of covalent attachment of a methyl group to the 5’ position of cytosine residues in CpG dinucleotides [56, 57]. CpG dinucleotides are not randomly distributed throughout the genome, but tend to cluster in regions called CpG islands, mainly present in the promoter region of the genes [54, 55, 57]. An accepted definition of CpG islands describes them as DNA sequences, more than 200 base pair long, with CG content greater than 50% and an observed/expected CpG ratio of more than 60% [54, 58]. Methylation can also occur at non-promoter CpG islands, defined as CpG shores, located in the vicinity of CpG islands up to 2 kb long [59, 60]. Methylation of CpG islands is typically associated with gene silencing, while demethylation of these sites enables transcription [54, 61]. Various risk factors like age, diet, chronic inflammation, infection with *H. Pylori* and EBV also act as a causative agent of aberrant gene methylation in GC [62].

Defective DNA methylation in *CDH1, CHFR, DAPK, GSTP1, p15, p16, RARβ, RASSF1A, RUNX3* and *TFPI2* has been considered as a serum biomarker for the diagnosis of GC [62, 63]. A large number of genes have been identified to be methylated in the gastric mucosa of GC patients. Among them, *RASGRF1* methylation has been found significantly elevated in mucosa from patients with either intestinal- or diffuse-type GC in comparison to mucosa from healthy individuals [64]. Silencing of miRNAs is also associated with hypermethylation of CpG islands. Methylation of the miR34-b/c was ubiquitous in GC cell lines but not in normal gastric mucosa from healthy *H. pylori*-negative individuals [65]. Aberrant DNA methylation in noncancerous gastric mucosa has been implicated in gastric carcinogenesis and could be a useful biomarker for the assessing risk of GC.

Multiple techniques are being used to identify aforementioned changes in the DNA methylation. Among them, pyrosequencing has been proved to be a more reliable method in comparison to both methylation specific polyme-

Hypermethylation in GPX3 promoter region with a 10% cut off was detected using pyrosequencing in 60% of the GC samples and 6 out of 9 cell lines [68]. Hypermethylation in *EDNRB* gene was analysed in 96 GC and adjacent normal tissues and correlated it with tumor infiltration [69]. Similarly, loss of expression of *FAT4* gene was observed in highly methylated GC cell lines and removal of methylation by demethylating agent restored its expression. Methylation status of *FAT4* has also been associated with *H. pylori* infection in GC [70]. The Cancer Genome Atlas (TCGA), by analysing 295 GC samples for CpG methylation level in 86 genes and 14 miRNAs, grouped hypermethylated genes into three categories: hypermethylated in EBV-positive subtype, hypermethylated in both EBV-positive and MSI-high subtypes, and other hypermethylated genes. Prominent methylation changes were observed in *RUNX1, ARHGDI B, PSME1, GZMB* and *RBM5* genes while *VAMP5* and *POLG* showed a marginal methylation difference between normal and GC cells.

The available literature documenting the role of epigenetic factors in the occurrence of gastric cancer clearly demonstrate the importance of strengthening efforts to pinpoint the key players that can be explored for the development of biomarkers and leads for better cancer management. A key advantage of NGS platforms is their ability to provide a comprehensive and unbiased view of the epigenome, facilitating investigations over content-limited microarray platforms.

**Differential gene expression in gastric cancer**

Study of differential gene expression in the normal versus tumor tissue provides important insights about the events governing the onset and progression of the disease. Information generated about the number and fold change of upregulated and downregulated genes during tumorigenesis may provide useful leads for further investigations aiming to identify relative importance of different pathways and key players participating in the disease progression. In
recent years, RNA-Seq approach has superseded the well-known microarray technique to an extent for assessing/computing of gene expression levels. Unlike microarrays, RNA-Seq can be used for the analysis of expression of novel transcripts without using probes.

Gene expression studies through NGS have been conducted using ovarian, colorectal and lung cancer specimens [71-73]. Transcriptome profiling of gastric tumor and normal tissues using Illumina sequencing revealed a total of 13,228 genes expressed in cancerous tissue in comparison to 13,674 genes expressed in normal tissue. Out of the expressed genes, 114 genes exhibited significant differential expression pattern between cancer and normal tissues with threshold false discovery rate (FDR) $<0.05$. CDH1 was the most significantly upregulated gene and its expression was surprisingly 309 times higher in cancer samples while DPT was the most downregulated gene showing 40 fold change. Dermatopontin gene (DPT) has been postulated to modify the behaviour of TGFBR2 through interaction with decorin and low expression was detected for both of these genes [10]. Another transcriptome profiling study in Chinese GC patients revealed 36 fold higher expression of CDH1 while DPT and TGFBR2 showed decreased expression in cancer samples [74] corroborating the earlier study [10]. The low expression of DPT in oral cancer has also been validated by qRT-PCR which substantiates the role of DPT as a common player in various cancers [75]. A study correlating gene expression and alteration pattern suggested that HER2 overexpression was in chorus with the ERBB2 amplification in 80% of the cases, while this phenomenon was exclusive and these patients did not have alterations in other receptor tyrosine kinases (RTKs) [29].

Length polymorphism at microsatellite loci in coding regions of genes may affect their expression by premature occurrence of stop codon. TGFBR2, a tumor suppressor gene, showed lack of expression in MSI-H samples. Expression of 139 genes with MSI in their UTR region was observed to be low when compared to genes without UTR mutations. Upregulated expression of 137 genes containing 210 mutations at microsatellite loci was observed and 96% of these mutations were present in the UTR regions. These observations suggest an influence of mutations in UTR on gene expression. Significant downregulated expression of MGLL, SORL1, C20orf194, WWC3, and PXDC1 genes was seen in MSI-H cell lines in contrast to MSS cell lines through transcriptome analysis and further validated by q-PCR. Mutations in 3’UTR region of MGLL gene resulted in 42.6% downregulation of recombinant luciferase indicating presence of aberrant gene products as a consequence of MSI. Deregulation of gene function in UTR could result from transcriptome altering mutations also [8].

Some studies have reported over expression of genes involved in receptor kinase activity. A tyrosine kinase receptor gene EGFR exhibited amplification and over expression in GC [76, 77]. Inhibitors of another gene of the RTK family, fibroblast growth factor receptor 2 (FGFR2), have shown some clinical efficacy in GC [11]. KI23057, one of the FGFR inhibitors, along with 5-fluorouracil has displayed synergistic antitumor effects for GC treatment [78]. Loss of function of SMAD4 gene helps in epithelial mesenchymal transition and its re-expression has been seen in reversing the process [79]. Expression of one of the important genes involved in breast cancer, BRCA1, is correlated with sensitivity to chemotherapeutics in gastric cancer [80, 81]. Silencing and overexpression of ARID1A gene led to both increased and decreased proliferation, respectively in tissue culture. Silencing of ARID1A gene also increases the level of E2F1 and cyclin E1 transcription factors. Long recurrence free survival has been predicted from mutation or deficiency of protein of ARID1A [20]. Expression of beta-catenin, FHIT, E-Cadherin, APC, CDX2, MET, TOP2A, HER2 and p53 has been investigated using FISH and immunohistochemistry. The results have suggested that beta-catenin, E-Cadherin and FHIT were among the highly expressed proteins. Expression of beta-catenin and E-cadherin was higher in patients with bad prognosis while FHIT was high in patients with good prognosis [23].

Liu and co-workers [37] have performed RNA-Seq analysis of 51 primary GC samples and 32 cell lines to study differential gene expression. SMTN, a smooth muscle expression marker, showed low expression in tumor as compared to normal tissue. One hundred and seventy differential isoform usage genes were identified including ZAK, KRAS, MCM7, ELK7 and CCND3 between tumor and normal gastric tissue. Sig-
A substantial amount of efforts have been directed to find a cure and develop better treatment regimes for different types of cancer. Still most of the generic therapies involve platinum and taxol based drugs, which despite their impressive success rates, also have severe side effects. Overall survival (OS) rate and quality of life post treatment by these chemotherapeutic agents is also low. This has led researchers to further look for disease and patient specific drugs with the major focus being on either activating the patient’s own immune system against the tumor cells or using the mutant and overexpressed protein specific antibodies. The insights gained from genetic and genomic studies on molecular pathogenesis of GC have prompted various studies aiming to identify different genetic biomarkers allowing early diagnosis and prognosis of the disease.

Classical biomarkers used for the diagnosis of GC include carcinoembryonic antigen (CEA) and cancer antigen 19-9 (CA-19-9), however, these biomarkers are not exclusive for GC and, therefore, their sensitivity and specificity is low. Other novel potential biomarkers based on DNA hypomethylation and miRNA are being explored for their applicability in screening for GC. The repertoire of prognostic GC markers based on MSI, CDH1, PI3K, KRAS, ALDH, SHH,
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Sox9, HER2, EGFR, VEGF, Hippo/YAP, MET targets show a detection rate varying from 4 to 40%, while some others like PD1, PDL are being considered promising futuristic markers requiring further validation [82, 83]. Development of these biomarkers has not only facilitated an early diagnosis of the disease but also played an important role in achieving recent advancements in the field of patient specific and targeted therapy. For example, trastuzumab, a HER2 specific monoclonal antibody is being used as a primary therapy in combination with chemotherapy. HER2 combinatorial drug has been shown to improve both quality of life and overall survival rate in HER2 mutation positive gastric cancers [84-86].

Table 3 presents an overview of the drugs marketed and under development for GC along with their mechanism of action. As can be inferred from the data available, most of the drugs under development are biological molecules, which act on the oncogenic cells either by activating the immune system or by inhibiting the proteins involved in metastasis and disease progression. Among the immunotherapeutic agents nivolumab and pembrolizumab have shown promising results in gastric cancer [90, 91]. These molecules target programmed cell death 1 (PD-1), which on interacting with PD-L1 causes suppression of the immune system. PD-1/PD-L1 related immune suppression and their expression level has also been associated with MSI+ GC [92, 93]. Obviously, many drugs undergo clinical trials but only a few clear the hurdles of accreditation. Different drugs have been grouped according to their nature and status of clinical phases as shown in Figure 5.

Genome wide association studies can help us understand the prevalence and identification of the specific therapies which could be delivered to the patients for better OS and quality of life. Different population and genetic studies have revealed various population specific mutations in GC. For example, PF-06671008 a bispecific anti-cadherin and anti-CD3 antibody, which is under clinical trials for breast cancer, colorectal cancer and non-small cell lung cancer [94] could also be used in treating GC patients with CDH1 mutations. CDH1 has also been identified as one of the prominent genetically transmitted gene for GC occurrence [95]. Several other studies based on genetic analysis of the GC patients have led to the identification of target for the development of patient specific drugs (https://ClinicalTrials.gov/show/NCT02-331693).

Conclusion

Comprehensive NGS-based studies on genetic and epigenetic changes, and differential gene
expression have generated enhanced thrust towards understanding different aspects of gastric tumorigenesis. Although, a plethora of genetic and epigenetic factors have been implicated, no consensus lines have evolved to define the molecular pathogenesis of gastric cancer. Nevertheless, a number of differentially expressed genes and genetic/epigenetic variants have been identified as potential targets for future investigations aiming to develop new biomarkers for early diagnosis of the disease. Moreover, new leads have been identified to assist the development of drugs to facilitate personalized therapy to complement patient specific treatment. The success of different NGS-based investigations in generating immensely useful information recently, will encourage researchers to undertake more extensive multidisciplinary efforts for better understanding of the events involved in the onset and progression of gastric cancer and identification of new targets for drug development.

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Disclosure of conflict of interest

None.

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NGS based molecular biology of gastric cancer


NGS based molecular biology of gastric cancer


