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Review Article

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

Renu Verma, Prakash C Sharma

University School of Biotechnology, Guru Gobind Singh Indraprastha University, New Delhi 110078, India

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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,

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

Sequencing approach	Sample source	Platform	Reference
Whole Genome Sequencing	Cell Lines	Ion Torrent/Illumina	[35]
	Cell Lines/Tissue	Illumina	[8]
	Tissue/Blood	Illumina	[25]
Exome Sequencing	Tissue	Illumina	[9]
	Tissue/Blood	Illumina	[96]
	Tissue	Illumina	[97]
Targeted Sequencing	Source undefined	Ion Torrent	[30]
	Source undefined	Illumina	[11]
	Tissue	Illumina	[40]
	Tissue	Ion Torrent	[18]
	Tissue/Blood	Ion Torrent	[34]
	Tissue	Ion Torrent	[29]
	Tissue	Ion Torrent	[98]
	Tissue	Ion Torrent	[41]
	Tissue	Illumina	[37]
	Tissue	Ion Torrent	[23]
	Tissue	Illumina	[8]
RNA-Seq	Tissue	Illumina	[99]
	Cell Lines/Tissue	Illumina	[100]
	Cell Lines	Illumina	[101]

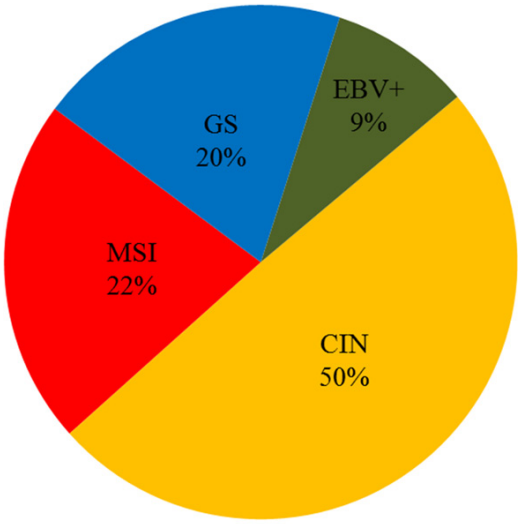

Figure 1. TCGA classification of different subtypes of gastric cancer.

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.

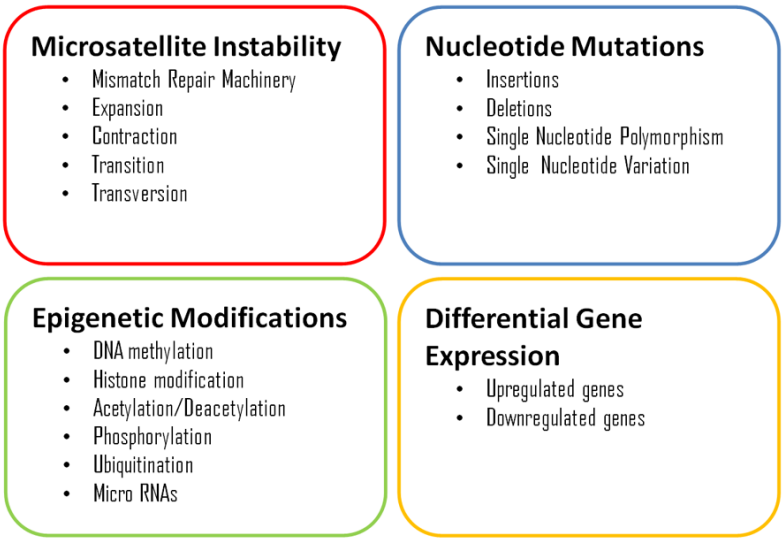

Figure 2. An outline representation of various molecular processes involved in gastric tumorigenesis.

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 10-10^4 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 impairs 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 frameshift 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 (SelTarbase), 24 candidate genes having deletion in their CDS were selected on the basis of driver gene score and pathway analysis, and subsequently

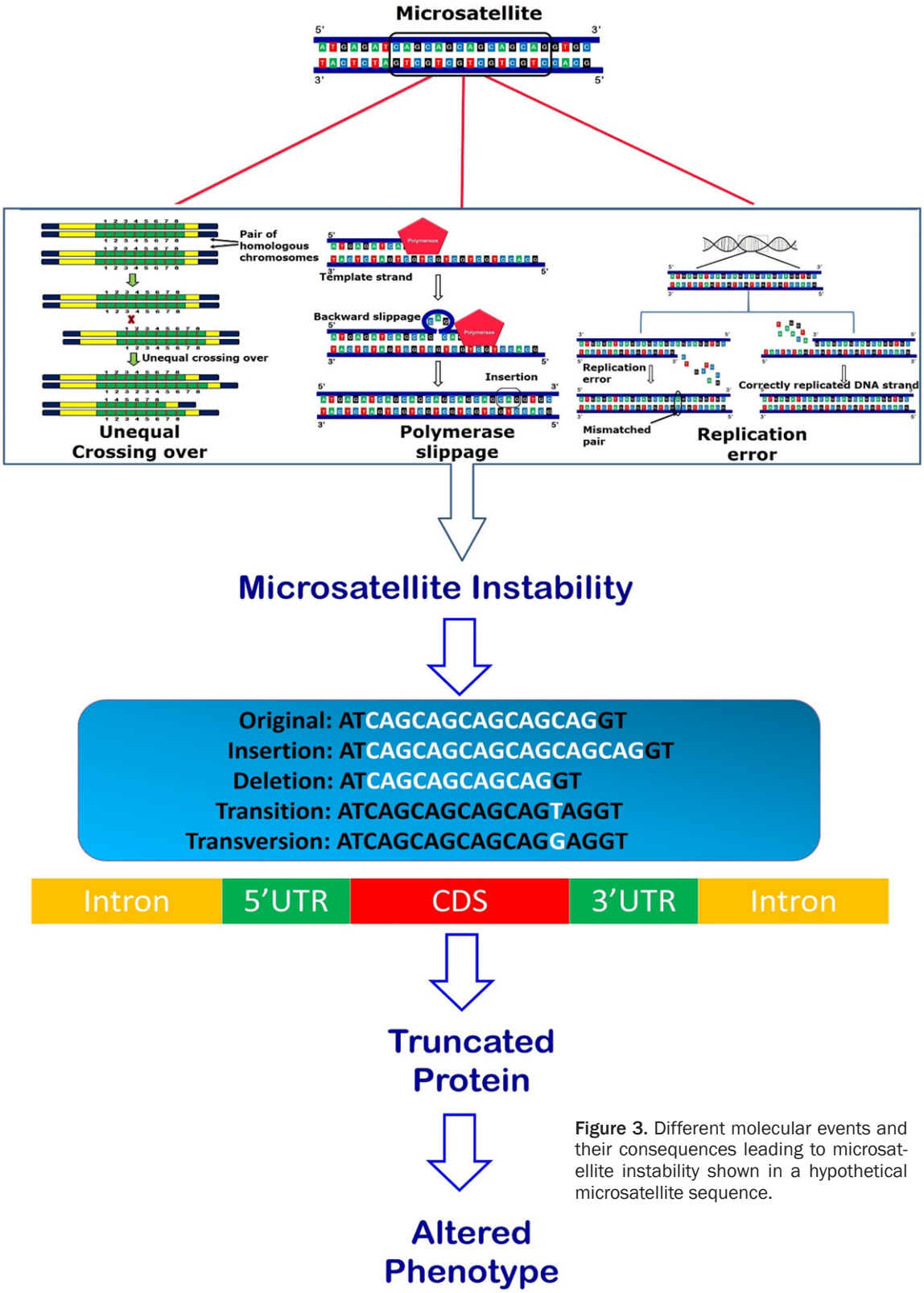

Figure 3. Different molecular events and their consequences leading to microsatellite instability shown in a hypothetical microsatellite sequence.

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 $(A)_{10}$ 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 instable GC patients by Nagarajan and colleagues [25] in 2012 found 14,856 somatic SNVs (11,738 InDels) in microsatellite instable sample and 17,473 somatic SNVs (2,486 InDels) in chromosomal instable 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

No. of patients	Target	Top mutated genes	Mutation (%)	Reference
121	409 genes	*TP53*	SNV (91.1) Deletion (6.7) Insertion (2.2)	[29]
		SYNE1	SNV (93.7) Insertion (6.2)	
		CSMD3	SNV (100)	
		LRP1B	SNV (100)	
		CDH1	SNV (81.81) Deletion (18.18)	
		PIK3CA	SNV (100)	
		ARID1A	SNV (45.4) Deletion (36.36) Insertion (18.18)	
		PKDH1	SNV (88.88) Insertion (11.11)	
		LPHN3	SNV (100)	
		MLL2	SNV (75) Deletion (25)	
		PRKDC	SNV (87.5) Insertion (12.5)	
		ERBB3	SNV (100)	
		ROS1	SNV (85.71) Deletion (14.28)	
		KAT6B	SNV (100)	
		PDE4DIP	SNV (66.66) Insertion (33.33)	
		RUNX1T1	SNV (100)	
22	Exome	*TP53*	SNV (36.36)	[21]
		PTEN	SNV (9) Indel (18)	
		ARID1A	SNV (9) Indel (18)	
		RPL22	Indel (13.6)	
		TTK	Indel (18)	
		FMN2	SNV (18)	
		SPRR2B	SNV (9)	
		PTN	SNV (4.5) Indel (4.5)	
		ACVR2A	Indel (18)	
		PMS2L3	SNV (4.5) Indel (4.5)	
		DNAH7	SNV (27.27) Indel (4.5)	
		TTN	SNV (22.72)	
		FSCB	SNV (13.63)	
		CTNNB1	SNV (9)	
		SEMA3E	SNV (9) Indel (4.5)	
		MCHR1	SNV (13.63)	
		SPANXN2	SNV (9)	
		METTL3	SNV (9)	
		EIF3A	SNV (13.6)	
		EPB41L3	SNV (9)	
15	Exome	*TP53*	SNV (73.3)	[20]
		DBR1	SNV (13)	
		RIT2	SNV (13)	
		CCNL1	SNV (13)	
		HTR1E	SNV (13)	
		ARID1A	SNV (20)	
		OR4C46	SNV (13)	
		OR4C15	SNV (13)	
		PIK3CA	SNV (20)	
		SHROOM3	SNV (13)	
20	50 genes	*KIT*	SNP (58)	[42]
		PDGFRA	SNP (26)	

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 *STAU2* 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 *STAU2* at $(T)_8$ MS locus were observed in 86%, 64% and 29% of MSI+ GC tumors, respectively. Mutations in *LMAN1* at $(T)_9$ 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 *TP-53, SYNE1, CSMD3, LRP1B, 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, respectively [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

by noncoding RNAs performs in harmony with *cis* and *trans* acting elements [53-55].

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 CG dinucleotides [56, 57]. CG 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*, *RAR*β, *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 polymerase chain reaction (MSP) and bisulfite sequencing [66]. In a comparative analysis, frequency of promoter region methylation in *TCF4* gene was reported to be higher when analyzed by pyrosequencing than MSP in advanced GC samples [67].

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, ARHGDIB, 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, TOPO2A, 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-

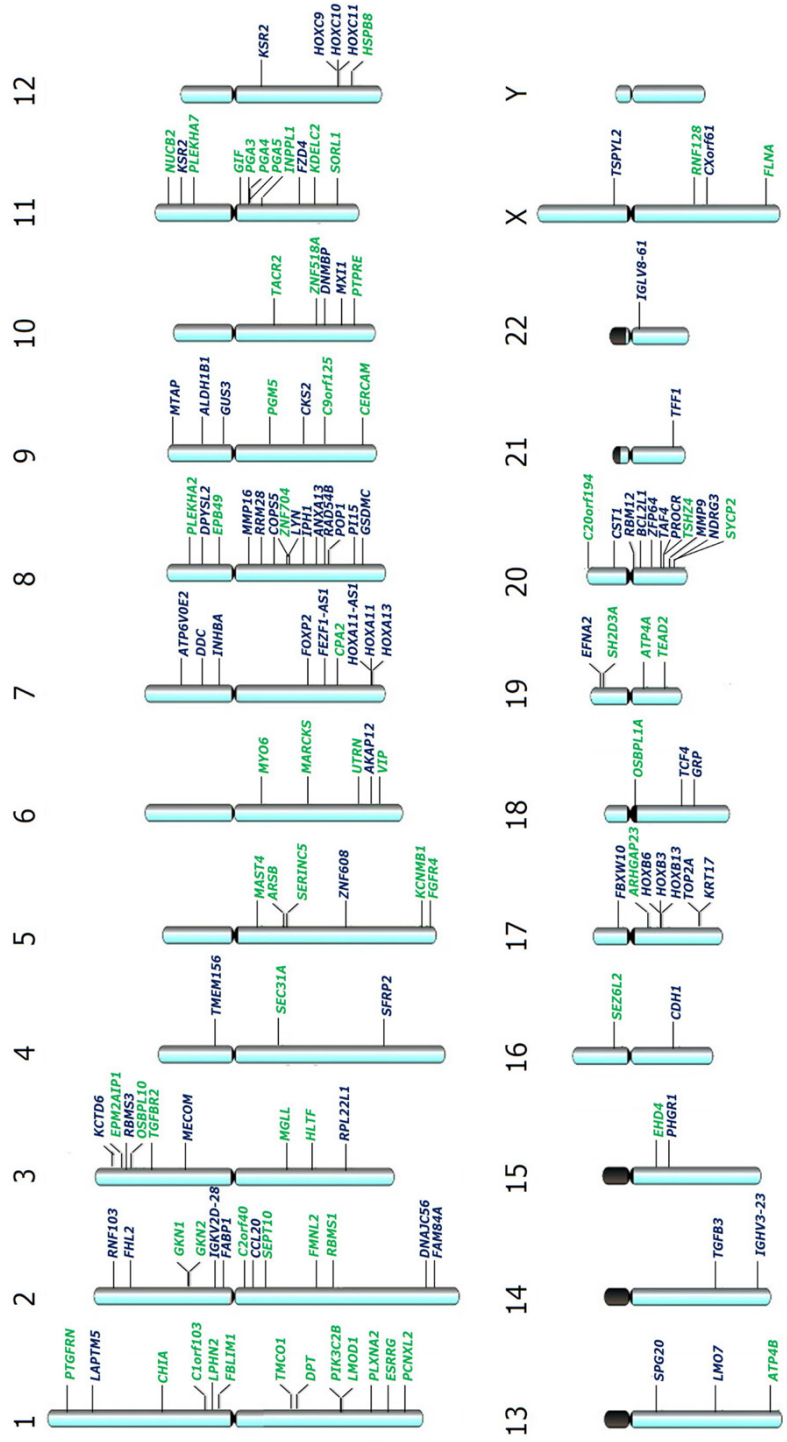

Figure 4. Important differentially expressed genes during gastric cancer and their chromosal positions. Upregulated genes are shown in green and down regulated genes are shown in blue.

nificant increase in the ZAK TV1 isoform fraction was observed in tumor samples while depletion of this isoform has been seen inhibiting proliferation in GC cell lines [37]. Important genes differentially regulated during GC and their chromosomal locations are shown in **Figure 4**.

Exploiting the leads for a better GC therapy

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,

Table 3. Details of drugs released/under trial for the treatment of gastric cancer

Phase	Drug	Product type	Target	Clinical trial/Drug bank	Source
Approved	Paclitaxel	Small molecule	Microtubules	DB01229	Taxusbrevifolia
Marketed	Apatinib mesylate	Small molecule	EGFR	http://advenchen.com/?page_id=13	Chemical synthesis
	Docetaxel	Small molecule	Microtubules	DB01248	Taxol derivative
	Doxorubicin	Small molecule	DNA intercalation	DB00997	Streptomyces
	Fluorouracil	Small molecule	Thymidylate synthase	DB00544	Semi-synthetic
	Mitomycin	Small molecule	DNA intercalation	DB00305	Streptomyces
	Ramucirumab	Monoclonal antibody	VEGFR-2	DB05578	Human
	Trastuzumab	Antibody drug conjugate	HER-2	DB00072	Antibody drug conjugate
Phase III	Bevacizumab	Monoclonal antibody	VEGF-A	NCT00887822	Humanized antibody
	Catumaxomab	Bispecific antibody; hybrid; rat-mouse	CD-3 and EpCAM	NCT00836654	Rat-mouse hybrid monoclonal antibody
	Everolimus	Small molecule	FKBP-12	NCT00879333	Semi-synthetic from Streptomyces hygroscopicus
	Lynparza	Small molecule	PARP	NCT01924533	Chemical synthesis
	Nimotuzumab	Monoclonal antibody	EGFR	NCT01813253	Humanized antibody
	Nivolumab	Biologic	PD-1	NCT03006705	Human
	Pembrolizumab	Monoclonal antibody	PD-1	NCT03019588	Humanized antibody
	Pertuzumab	Monoclonal antibody	HER-2	NCT01774786	Humanized antibody
Phase II	Afatinib	Small molecule	Mutant EGFR	NCT02501603	Chemical synthesis
	Alpelisib	Small molecule	PI3k	NCT01708161	Chemical synthesis
	AMG 337	Small molecule	Hepatocyte growth factor receptor	NCT02016534	Chemical synthesis
	Atezolizumab	Biologic	PD-L1	NCT02458638	Humanized antibody
	AZD4547	Small molecule	FGFR	NCT01795768	Chemical synthesis
	Cabazitaxel	Small molecule	Microtubules	NCT01956149	Taxoid derivative
	Camptothecin	Small molecule	DNA topoisomerase 1	NCT00080002	Camptothecaacuminata
	Dovitinib lactate	Small molecule	Receptor tyrosine kinase	NCT01478373	Chemical synthesis
	Durvalumab	Monoclonal antibody	PD-L1	NCT03094286	Human
	GlutaDON	Small molecule	Glutamate analogue		Semi-synthetic
	Ipatasertib	Small molecule	Akt	NCT01896531	Chemical synthesis
Phase II	Ipilimumab	Monoclonal antibody	CTLA-4	NCT02935634	Human
	Luminespib	Small molecule	Hsp90	NCT01084330	Chemical synthesis
	LY-2875358	Monoclonal antibody	Hepatocyte growth factor receptor	NCT01874938	Humanized antibody
	Masitinib	Small molecule	Receptor tyrosine kinase	NCT01506336	Chemical synthesis
	MM-111	Bi specific antibody	HER-2 and HER-3	NCT01774851	Human serum albumin based antibody
	Mogamulizumab	Biologic	C-C chemokine receptor 4	NCT02281409	Humanized antibody
	Neratinib	Small molecule	HER-2 and EGFR	NCT01953926	Chemical synthesis
	Oxaliplatin	Small molecule; Monoclonal antibody	DNA intercalation	NCT01980407	Chemical synthesis
	Poziotinib	Small molecule	EGFR	NCT01746771	Chemical synthesis
	Regorafenib	Small molecule	Receptor tyrosine kinase	NCT01913639	Chemical synthesis
	Sacituzumab govitecan	Antibody drug conjugate	TROP-2	NCT01631552	Semi-synthetic
	Tasquinimod	Small molecule	S100 calcium-binding protein A9	NCT01743469	Chemical synthesis
	Telatinib	Small molecule	Receptor tyrosine kinase	NCT00952497	Chemical synthesis
	Tivantinib	Small molecule	Hepatocyte growth factor receptor	NCT01152645	Chemical synthesis
	Tremelimumab	Monoclonal antibody	CTLA-4	NCT02340975	Human
	Varlitinib tosylate	Small molecule	HER-2 and EGFR	http://www.arraybiopharma.com/product-pipeline/other-compounds/aslan001-arry-543/	Chemical synthesis

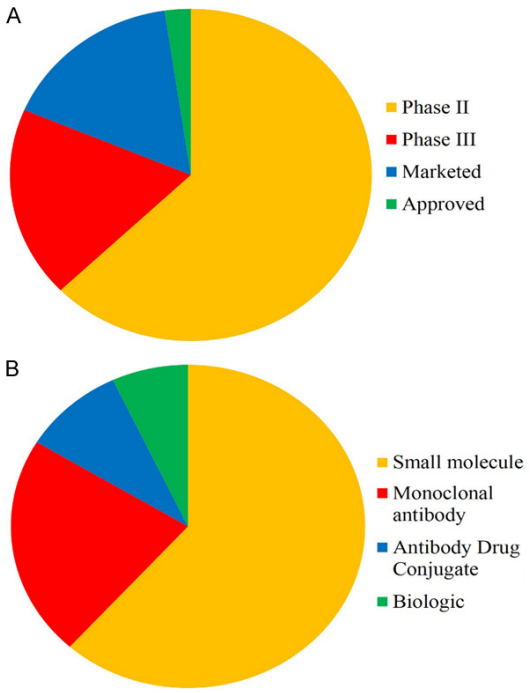

Figure 5. Relative distribution of drugs according to clinical phases (A) and product type (B) developed for the treatment of gastric cancer.

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].

The success of overexpression of specific antibodies approach can be seen by the development and use of trastuzumab, a HER2 specific monoclonal antibody, approved specifically for HER2 overexpressing GC patients, in combination with 5-flurouracil or capecitabine. Adding trastuzumab to chemotherapy regime has improved median survival of GC patients by 2.5 months. The combination therapy also showed an enhancement in progression free survival (PFS) and overall response rate by 6.7 months versus 5.5 months and 47.3% vs. 34.5% over chemotherapy alone [87]. This positive result of trastuzumab treatment, has also led to its inclusion in National Comprehensive Cancer Network (NCCN) guidelines for a standard care therapy [88]. Similarly, ramucirumab, a VEGFR2 inhibitor, has also received approval from FDA for metastatic gastric cancer after showing an increase in OS and PFS in comparison to placebo [89]. **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 of gastric tumorigenesis. Although, a pleothera 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.

Address correspondence to: Prakash C Sharma, University School of Biotechnology, Guru Gobind Singh Indraprastha University, New Delhi 110078, India. E-mail: prof.pcsharma@gmail.com

References

[1] Siegel R, Ma J, Zou Z and Jemal A. Cancer statistics, 2014. CA Cancer J Clin 2014; 64: 9-29.
[2] Lauren P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt at a histo-clinical classification. Acta Pathol Microbiol Scand 1965; 64: 31-49.
[3] Ma J, Shen H, Kapesa L and Zeng S. Lauren classification and individualized chemotherapy in gastric cancer. Oncol Lett 2016; 11: 2959-2964.
[4] Cheng XJ, Lin JC and Tu SP. Etiology and prevention of gastric cancer. Gastrointest Tumors 2016; 3: 25-36.
[5] Behjati S and Tarpey PS. What is next generation sequencing? Arch Dis Child Educ Pract Ed 2013; 98: 236-238.
[6] Shendure J and Ji H. Next-generation DNA sequencing. Nat Biotechnol 2008; 26: 1135-1145.
[7] Serrati S, De Summa S, Pilato B, Petriella D, Lacalamita R, Tommasi S and Pinto R. Next-generation sequencing: advances and applications in cancer diagnosis. Onco Targets Ther 2016; 9: 7355-7365.
[8] Yoon K, Lee S, Han TS, Moon SY, Yun SM, Kong SH, Jho S, Choe J, Yu J, Lee HJ, Park JH, Kim HM, Lee SY, Park J, Kim WH, Bhak J, Yang HK and Kim SJ. Comprehensive genome- and transcriptome-wide analyses of mutations associated with microsatellite instability in Korean gastric cancers. Genome Res 2013; 23: 1109-1117.
[9] Shimizu T, Marusawa H, Matsumoto Y, Inuzuka T, Ikeda A, Fujii Y, Minamiguchi S, Miyamoto S, Kou T, Sakai Y, Crabtree JE and Chiba T. Accumulation of somatic mutations in TP53 in gastric epithelium with helicobacter pylori infection. Gastroenterology 2014; 147: 407-417, e403.
[10] Zhang FG, He ZY and Wang Q. Transcriptome profiling of the cancer and normal tissues from gastric cancer patients by deep sequencing. Tumour Biol 2014; 35: 7423-7427.
[11] Holbrook JD, Parker JS, Gallagher KT, Halsey WS, Hughes AM, Weigman VJ, Lebowitz PF and Kumar R. Deep sequencing of gastric carcinoma reveals somatic mutations relevant to personalized medicine. J Transl Med 2011; 9: 119.
[12] van Dijk EL, Auger H, Jaszczyszyn Y and Thermes C. Ten years of next-generation sequencing technology. Trends Genet 2014; 30: 418-426.
[13] Cancer Genome Atlas Research Network. Comprehensive molecular characterization of gastric adenocarcinoma. Nature 2014; 513: 202-209.
[14] Velho S, Fernandes MS, Leite M, Figueiredo C and Seruca R. Causes and consequences of microsatellite instability in gastric carcinogenesis. World J Gastroenterol 2014; 20: 16433-16442.
[15] Shokal U and Sharma PC. Implication of microsatellite instability in human gastric cancers. Indian J Med Res 2012; 135: 599-613.
[16] Ropero S, Fraga MF, Ballestar E, Hamelin R, Yamamoto H, Boix-Chornet M, Caballero R, Alaminos M, Setien F, Paz MF, Herranz M, Palacios J, Arango D, Orntoft TF, Aaltonen LA, Schwartz S Jr and Esteller M. A truncating mutation of HDAC2 in human cancers confers re-

sistance to histone deacetylase inhibition. Nat Genet 2006; 38: 566-569.
[17] Melo SA, Moutinho C, Ropero S, Calin GA, Rossi S, Spizzo R, Fernandez AF, Davalos V, Villanueva A, Montoya G, Yamamoto H, Schwartz S Jr and Esteller M. A genetic defect in exportin-5 traps precursor microRNAs in the nucleus of cancer cells. Cancer Cell 2010; 18: 303-315.
[18] Kim TM, Laird PW and Park PJ. The landscape of microsatellite instability in colorectal and endometrial cancer genomes. Cell 2013; 155: 858-868.
[19] Yamamoto H, Watanabe Y, Maehata T, Morita R, Yoshida Y, Oikawa R, Ishigooka S, Ozawa S, Matsuo Y, Hosoya K, Yamashita M, Taniguchi H, Nosho K, Suzuki H, Yasuda H, Shinomura Y and Itoh F. An updated review of gastric cancer in the next-generation sequencing era: insights from bench to bedside and vice versa. World J Gastroenterol 2014; 20: 3927-3937.
[20] Zang ZJ, Cutcutache I, Poon SL, Zhang SL, McPherson JR, Tao J, Rajasegaran V, Heng HL, Deng N, Gan A, Lim KH, Ong CK, Huang D, Chin SY, Tan IB, Ng CC, Yu W, Wu Y, Lee M, Wu J, Poh D, Wan WK, Rha SY, So J, Salto-Tellez M, Yeoh KG, Wong WK, Zhu YJ, Futreal PA, Pang B, Ruan Y, Hillmer AM, Bertrand D, Nagarajan N, Rozen S, Teh BT and Tan P. Exome sequencing of gastric adenocarcinoma identifies recurrent somatic mutations in cell adhesion and chromatin remodeling genes. Nat Genet 2012; 44: 570-574.
[21] Wang K, Kan J, Yuen ST, Shi ST, Chu KM, Law S, Chan TL, Kan Z, Chan AS, Tsui WY, Lee SP, Ho SL, Chan AK, Cheng GH, Roberts PC, Rejto PA, Gibson NW, Pocalyko DJ, Mao M, Xu J and Leung SY. Exome sequencing identifies frequent mutation of ARID1A in molecular subtypes of gastric cancer. Nat Genet 2011; 43: 1219-1223.
[22] Greenman C, Stephens P, Smith R, Dalgliesh GL, Hunter C, Bignell G, Davies H, Teague J, Butler A, Stevens C, Edkins S, O'Meara S, Vastrik I, Schmidt EE, Avis T, Barthorpe S, Bhamra G, Buck G, Choudhury B, Clements J, Cole J, Dicks E, Forbes S, Gray K, Halliday K, Harrison R, Hills K, Hinton J, Jenkinson A, Jones D, Menzies A, Mironenko T, Perry J, Raine K, Richardson D, Shepherd R, Small A, Tofts C, Varian J, Webb T, West S, Widaa S, Yates A, Cahill DP, Louis DN, Goldstraw P, Nicholson AG, Brasseur F, Looijenga L, Weber BL, Chiew YE, DeFazio A, Greaves MF, Green AR, Campbell P, Birney E, Easton DF, Chenevix-Trench G, Tan MH, Khoo SK, Teh BT, Yuen ST, Leung SY, Wooster R, Futreal PA and Stratton MR. Patterns of somatic mutation in human cancer genomes. Nature 2007; 446: 153-158.
[23] Bria E, Pilotto S, Simbolo M, Fassan M, de Manzoni G, Carbognin L, Sperduti I, Brunelli M, Cataldo I, Tomezzoli A, Mafficini A, Turri G, Karachaliou N, Rosell R, Tortora G and Scarpa A. Comprehensive molecular portrait using next generation sequencing of resected intestinal-type gastric cancer patients dichotomized according to prognosis. Sci Rep 2016; 6: 22982.
[24] Sukawa Y, Yamamoto H, Nosho K, Kunimoto H, Suzuki H, Adachi Y, Nakazawa M, Nobuoka T, Kawayama M, Mikami M, Matsuno T, Hasegawa T, Hirata K, Imai K and Shinomura Y. Alterations in the human epidermal growth factor receptor 2-phosphatidylinositol 3-kinase-v-Akt pathway in gastric cancer. World J Gastroenterol 2012; 18: 6577-6586.
[25] Nagarajan N, Bertrand D, Hillmer AM, Zang ZJ, Yao F, Jacques PE, Teo AS, Cutcutache I, Zhang Z, Lee WH, Sia YY, Gao S, Ariyaratne PN, Ho A, Woo XY, Veeravali L, Ong CK, Deng N, Desai KV, Khor CC, Hibberd ML, Shahab A, Rao J, Wu M, Teh M, Zhu F, Chin SY, Pang B, So JB, Bourque G, Soong R, Sung WK, Tean Teh B, Rozen S, Ruan X, Yeoh KG, Tan PB and Ruan Y. Whole-genome reconstruction and mutational signatures in gastric cancer. Genome Biol 2012; 13: R115.
[26] Woerner SM, Yuan YP, Benner A, Korff S, von Knebel Doeberitz M and Bork P. SelTarbase, a database of human mononucleotide-microsatellite mutations and their potential impact to tumorigenesis and immunology. Nucleic Acids Res 2010; 38: D682-689.
[27] Hempen PM, Zhang L, Bansal RK, Iacobuzio-Donahue CA, Murphy KM, Maitra A, Vogelstein B, Whitehead RH, Markowitz SD, Willson JK, Yeo CJ, Hruban RH and Kern SE. Evidence of selection for clones having genetic inactivation of the activin A type II receptor (ACVR2) gene in gastrointestinal cancers. Cancer Res 2003; 63: 994-999.
[28] Jung B, Doctolero RT, Tajima A, Nguyen AK, Keku T, Sandler RS and Carethers JM. Loss of activin receptor type 2 protein expression in microsatellite unstable colon cancers. Gastroenterology 2004; 126: 654-659.
[29] Kuboki Y, Yamashita S, Niwa T, Ushijima T, Nagatsuma A, Kuwata T, Yoshino T, Doi T, Ochiai A and Ohtsu A. Comprehensive analyses using next-generation sequencing and immunohistochemistry enable precise treatment in advanced gastric cancer. Ann Oncol 2016; 27: 127-133.
[30] El-Husny A, Raiol-Moraes M, Amador M, Ribeiro-Dos-Santos AM, Montagnini A, Barbosa S, Silva A, Assumpcao P, Ishak G, Santos S, Pinto P, Cruz A and Ribeiro-Dos-Santos A. CDH1 mutations in gastric cancer patients

from northern Brazil identified by next- generation sequencing (NGS). Genet Mol Biol 2016; 39: 189-198.
[31] Guilford P, Humar B and Blair V. Hereditary diffuse gastric cancer: translation of CDH1 germline mutations into clinical practice. Gastric Cancer 2010; 13: 1-10.
[32] Lajus TB and Sales RM. CDH1 germ-line missense mutation identified by multigene sequencing in a family with no history of diffuse gastric cancer. Gene 2015; 568: 215-219.
[33] Risinger JI, Berchuck A, Kohler MF and Boyd J. Mutations of the E-cadherin gene in human gynecologic cancers. Nat Genet 1994; 7: 98-102.
[34] Hirotsu Y, Kojima Y, Okimoto K, Amemiya K, Mochizuki H and Omata M. Comparison between two amplicon-based sequencing panels of different scales in the detection of somatic mutations associated with gastric cancer. BMC Genomics 2016; 17: 833.
[35] Hou Y, Wu K, Shi X, Li F, Song L, Wu H, Dean M, Li G, Tsang S, Jiang R, Zhang X, Li B, Liu G, Bedekar N, Lu N, Xie G, Liang H, Chang L, Wang T, Chen J, Li Y, Zhang X, Yang H, Xu X, Wang L and Wang J. Comparison of variations detection between whole-genome amplification methods used in single-cell resequencing. Gigascience 2015; 4: 37.
[36] Bose R, Kavuri SM, Searleman AC, Shen W, Shen D, Koboldt DC, Monsey J, Goel N, Aronson AB, Li S, Ma CX, Ding L, Mardis ER and Ellis MJ. Activating HER2 mutations in HER2 gene amplification negative breast cancer. Cancer Discov 2013; 3: 224-237.
[37] Liu J, McCleland M, Stawiski EW, Gnad F, Mayba O, Haverty PM, Durinck S, Chen YJ, Klijn C, Jhunjhunwala S, Lawrence M, Liu H, Wan Y, Chopra V, Yaylaoglu MB, Yuan W, Ha C, Gilbert HN, Reeder J, Pau G, Stinson J, Stern HM, Manning G, Wu TD, Neve RM, de Sauvage FJ, Modrusan Z, Seshagiri S, Firestein R and Zhang Z. Integrated exome and transcriptome sequencing reveals ZAK isoform usage in gastric cancer. Nat Commun 2014; 5: 3830.
[38] Zang ZJ, Ong CK, Cutcutache I, Yu W, Zhang SL, Huang D, Ler LD, Dykema K, Gan A, Tao J, Lim S, Liu Y, Futreal PA, Grabsch H, Furge KA, Goh LK, Rozen S, Teh BT and Tan P. Genetic and structural variation in the gastric cancer kinome revealed through targeted deep sequencing. Cancer Res 2011; 71: 29-39.
[39] Li X, Wu WK, Xing R, Wong SH, Liu Y, Fang X, Zhang Y, Wang M, Wang J, Li L, Zhou Y, Tang S, Peng S, Qiu K, Chen L, Chen K, Yang H, Zhang W, Chan MT, Lu Y, Sung JJ and Yu J. Distinct subtypes of gastric cancer defined by molecular characterization include novel mutational signatures with prognostic capability. Cancer Res 2016; 76: 1724-1732.
[40] Fisher KE, Zhang L, Wang J, Smith GH, Newman S, Schneider TM, Pillai RN, Kudchadkar RR, Owonikoko TK, Ramalingam SS, Lawson DH, Delman KA, El-Rayes BF, Wilson MM, Sullivan HC, Morrison AS, Balci S, Adsay NV, Gal AA, Sica GL, Saxe DF, Mann KP, Hill CE, Khuri FR and Rossi MR. Clinical validation and implementation of a targeted next-generation sequencing assay to detect somatic variants in non-small cell lung, melanoma, and gastrointestinal malignancies. J Mol Diagn 2016; 18: 299-315.
[41] Xu HY, Xu WL, Wang LQ, Chen MB and Shen HL. Relationship between p53 status and response to chemotherapy in patients with gastric cancer: a meta-analysis. PLoS One 2014; 9: e95371.
[42] Gleeson FC, Kipp BR, Kerr SE, Voss JS, Graham RP, Campion MB, Minot DM, Tu ZJ, Klee EW, Lazaridis KN, Henry MR and Levy MJ. Kinase genotype analysis of gastric gastrointestinal stromal tumor cytology samples using targeted next-generation sequencing. Clin Gastroenterol Hepatol 2015; 13: 202-206.
[43] Mafficini A, Amato E, Fassan M, Simbolo M, Antonello D, Vicentini C, Scardoni M, Bersani S, Gottardi M, Rusev B, Malpeli G, Corbo V, Barbi S, Sikora KO, Lawlor RT, Tortora G and Scarpa A. Reporting tumor molecular heterogeneity in histopathological diagnosis. PLoS One 2014; 9: e104979.
[44] Kakiuchi M, Nishizawa T, Ueda H, Gotoh K, Tanaka A, Hayashi A, Yamamoto S, Tatsuno K, Katoh H, Watanabe Y, Ichimura T, Ushiku T, Funahashi S, Tateishi K, Wada I, Shimizu N, Nomura S, Koike K, Seto Y, Fukayama M, Aburatani H and Ishikawa S. Recurrent gain-of-function mutations of RHOA in diffuse-type gastric carcinoma. Nat Genet 2014; 46: 583-587.
[45] Berger SL, Kouzarides T, Shiekhattar R and Shilatifard A. An operational definition of epigenetics. Genes Dev 2009; 23: 781-783.
[46] Kang C, Song JJ, Lee J and Kim MY. Epigenetics: an emerging player in gastric cancer. World J Gastroenterol 2014; 20: 6433-6447.
[47] Handel AE, Ebers GC and Ramagopalan SV. Epigenetics: molecular mechanisms and implications for disease. Trends Mol Med 2010; 16: 7-16.
[48] Heyn H, Moran S, Hernando-Herraez I, Sayols S, Gomez A, Sandoval J, Monk D, Hata K, Marques-Bonet T, Wang L and Esteller M. DNA methylation contributes to natural human variation. Genome Res 2013; 23: 1363-1372.
[49] Baylin SB and Jones PA. A decade of exploring the cancer epigenome-biological and translational implications. Nat Rev Cancer 2011; 11: 726-734.
[50] Dawson MA and Kouzarides T. Cancer epigenetics: from mechanism to therapy. Cell 2012; 150: 12-27.
[51] Schuebel KE, Chen W, Cope L, Glockner SC, Suzuki H, Yi JM, Chan TA, Van Neste L, Van

Criekinge W, van den Bosch S, van Engeland M, Ting AH, Jair K, Yu W, Toyota M, Imai K, Ahuja N, Herman JG and Baylin SB. Comparing the DNA hypermethylome with gene mutations in human colorectal cancer. PLoS Genet 2007; 3: 1709-1723.

[52] You JS and Jones PA. Cancer genetics and epigenetics: two sides of the same coin? Cancer Cell 2012; 22: 9-20.

[53] Ellis L, Atadja PW and Johnstone RW. Epigenetics in cancer: targeting chromatin modifications. Mol Cancer Ther 2009; 8: 1409-1420.

[54] Sandoval J and Esteller M. Cancer epigenomics: beyond genomics. Curr Opin Genet Dev 2012; 22: 50-55.

[55] Sandoval J, Peiro-Chova L, Pallardo FV and Garcia-Gimenez JL. Epigenetic biomarkers in laboratory diagnostics: emerging approaches and opportunities. Expert Rev Mol Diagn 2013; 13: 457-471.

[56] Mazzio EA and Soliman KF. Basic concepts of epigenetics: impact of environmental signals on gene expression. Epigenetics 2012; 7: 119-130.

[57] Korkmaz A, Manchester LC, Topal T, Ma S, Tan DX and Reiter RJ. Epigenetic mechanisms in human physiology and diseases. J Exp Integr Med 2011; 1: 139-147.

[58] Kim JK, Samaranayake M and Pradhan S. Epigenetic mechanisms in mammals. Cell Mol Life Sci 2009; 66: 596-612.

[59] Doi A, Park IH, Wen B, Murakami P, Aryee MJ, Irizarry R, Herb B, Ladd-Acosta C, Rho J, Loewer S, Miller J, Schlaeger T, Daley GQ and Feinberg AP. Differential methylation of tissue- and cancer-specific CpG island shores distinguishes human induced pluripotent stem cells, embryonic stem cells and fibroblasts. Nat Genet 2009; 41: 1350-1353.

[60] Irizarry RA, Ladd-Acosta C, Wen B, Wu Z, Montano C, Onyango P, Cui H, Gabo K, Rongione M, Webster M, Ji H, Potash JB, Sabunciyan S and Feinberg AP. The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. Nat Genet 2009; 41: 178-186.

[61] Inbar-Feigenberg M, Choufani S, Butcher DT, Roifman M and Weksberg R. Basic concepts of epigenetics. Fertil Steril 2013; 99: 607-615.

[62] Qu Y, Dang S and Hou P. Gene methylation in gastric cancer. Clin Chim Acta 2013; 424: 53-65.

[63] Sapari NS, Loh M, Vaithilingam A and Soong R. Clinical potential of DNA methylation in gastric cancer: a meta-analysis. PLoS One 2012; 7: e36275.

[64] Takamaru H, Yamamoto E, Suzuki H, Nojima M, Maruyama R, Yamano HO, Yoshikawa K, Kimura T, Harada T, Ashida M, Suzuki R, Yamamoto H, Kai M, Tokino T, Sugai T, Imai K, Toyota M and Shinomura Y. Aberrant methylation of RASGRF1 is associated with an epigenetic field defect and increased risk of gastric cancer. Cancer Prev Res (Phila) 2012; 5: 1203-1212.

[65] Suzuki H, Yamamoto E, Nojima M, Kai M, Yamano HO, Yoshikawa K, Kimura T, Kudo T, Harada E, Sugai T, Takamaru H, Niinuma T, Maruyama R, Yamamoto H, Tokino T, Imai K, Toyota M and Shinomura Y. Methylation-associated silencing of microRNA-34b/c in gastric cancer and its involvement in an epigenetic field defect. Carcinogenesis 2010; 31: 2066-2073.

[66] Reed K, Poulin ML, Yan L and Parissenti AM. Comparison of bisulfite sequencing PCR with pyrosequencing for measuring differences in DNA methylation. Anal Biochem 2010; 397: 96-106.

[67] Joo JK, Kim SH, Kim HG, Kim DY, Ryu SY, Lee KH and Lee JH. CpG methylation of transcription factor 4 in gastric carcinoma. Ann Surg Oncol 2010; 17: 3344-3353.

[68] Peng DF, Hu TL, Schneider BG, Chen Z, Xu ZK and El-Rifai W. Silencing of glutathione peroxidase 3 through DNA hypermethylation is associated with lymph node metastasis in gastric carcinomas. PLoS One 2012; 7: e46214.

[69] Tao K, Wu C, Wu K, Li W, Han G, Shuai X and Wang G. Quantitative analysis of promoter methylation of the EDNRB gene in gastric cancer. Med Oncol 2012; 29: 107-112.

[70] Yoshida S, Yamashita S, Niwa T, Mori A, Ito S, Ichinose M and Ushijima T. Epigenetic inactivation of FAT4 contributes to gastric field cancerization. Gastric Cancer 2017; 20: 136-145.

[71] Jazaeri AA, Yee CJ, Sotiriou C, Brantley KR, Boyd J and Liu ET. Gene expression profiles of BRCA1-linked, BRCA2-linked, and sporadic ovarian cancers. J Natl Cancer Inst 2002; 94: 990-1000.

[72] Koh KH, Rhee H, Kang HJ, Yang E, You KT, Lee H, Min BS, Kim NK, Nam SW and Kim H. Differential gene expression profiles of metastases in paired primary and metastatic colorectal carcinomas. Oncology 2008; 75: 92-101.

[73] Valk K, Vooder T, Kolde R, Reintam MA, Petzold C, Vilo J and Metspalu A. Gene expression profiles of non-small cell lung cancer: survival prediction and new biomarkers. Oncology 2010; 79: 283-292.

[74] Wu HQ, Wang HY, Sun XW, Liu F, Zhang LW and Tian FJ. Transcriptome profiling of cancers tissue in Chinese gastric patients by high-through sequencing. Int J Clin Exp Pathol 2016; 9: 3537-3546.

[75] Yamatoji M, Kasamatsu A, Kouzu Y, Koike H, Sakamoto Y, Ogawara K, Shiiba M, Tanzawa H

and Uzawa K. Dermatopontin: a potential predictor for metastasis of human oral cancer. Int J Cancer 2012; 130: 2903-2911.
[76] Arkenau HT. Gastric cancer in the era of molecularly targeted agents: current drug development strategies. J Cancer Res Clin Oncol 2009; 135: 855-866.
[77] Ku GY and Ilson DH. Esophagogastric cancer: targeted agents. Cancer Treat Rev 2010; 36: 235-248.
[78] Yashiro M, Shinto O, Nakamura K, Tendo M, Matsuoka T, Matsuzaki T, Kaizaki R, Miwa A and Hirakawa K. Synergistic antitumor effects of FGFR2 inhibitor with 5-fluorouracil on scirrhous gastric carcinoma. Int J Cancer 2010; 126: 1004-1016.
[79] Pohl M, Radacz Y, Pawlik N, Schoeneck A, Baldus SE, Munding J, Schmiegel W, Schwarte-Waldhoff I and Reinacher-Schick A. SMAD4 mediates mesenchymal-epithelial reversion in SW480 colon carcinoma cells. Anticancer Res 2010; 30: 2603-2613.
[80] Shim HJ, Yun JY, Hwang JE, Bae WK, Cho SH, Lee JH, Kim HN, Shin MH, Kweon SS, Lee JH, Kim HJ and Chung IJ. BRCA1 and XRCC1 polymorphisms associated with survival in advanced gastric cancer treated with taxane and cisplatin. Cancer Sci 2010; 101: 1247-1254.
[81] Wang L, Wei J, Qian X, Yin H, Zhao Y, Yu L, Wang T and Liu B. ERCC1 and BRCA1 mRNA expression levels in metastatic malignant effusions is associated with chemosensitivity to cisplatin and/or docetaxel. BMC Cancer 2008; 8: 97.
[82] Elimova E, Wadhwa R, Shiozaki H, Sudo K, Estrella JS, Badgwell BD, Das P, Matamoros A Jr, Song S and Ajani JA. Molecular biomarkers in gastric cancer. J Natl Compr Canc Netw 2015; 13: e19-29.
[83] Duraes C, Almeida GM, Seruca R, Oliveira C and Carneiro F. Biomarkers for gastric cancer: prognostic, predictive or targets of therapy? Virchows Arch 2014; 464: 367-378.
[84] Bang YJ, Van Cutsem E, Feyereislova A, Chung HC, Shen L, Sawaki A, Lordick F, Ohtsu A, Omuro Y, Satoh T, Aprile G, Kulikov E, Hill J, Lehle M, Ruschoff J, Kang YK; ToGA Trial Investigators. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. Lancet 2010; 376: 687-697.
[85] Gong J, Liu T, Fan Q, Bai L, Bi F, Qin S, Wang J, Xu N, Cheng Y, Bai Y, Liu W, Wang L and Shen L. Optimal regimen of trastuzumab in combination with oxaliplatin/capecitabine in first-line treatment of HER2-positive advanced gastric cancer (CGOG1001): a multicenter, phase II trial. BMC Cancer 2016; 16: 68.
[86] Sanford M. Trastuzumab: a review of its use in HER2-positive advanced gastric cancer. Drugs 2013; 73: 1605-1615.
[87] Gunturu KS, Woo Y, Beaubier N, Remotti HE and Saif MW. Gastric cancer and trastuzumab: first biologic therapy in gastric cancer. Ther Adv Med Oncol 2013; 5: 143-151.
[88] Ajani JA, D'Amico TA, Almhanna K, Bentrem DJ, Chao J, Das P, Denlinger CS, Fanta P, Farjah F, Fuchs CS, Gerdes H, Gibson M, Glasgow RE, Hayman JA, Hochwald S, Hofstetter WL, Ilson DH, Jaroszewski D, Johung KL, Keswani RN, Kleinberg LR, Korn WM, Leong S, Linn C, Lockhart AC, Ly QP, Mulcahy MF, Orringer MB, Perry KA, Poultsides GA, Scott WJ, Strong VE, Washington MK, Weksler B, Willett CG, Wright CD, Zelman D, McMillian N and Sundar H. Gastric cancer, version 3.2016, NCCN clinical practice guidelines in oncology. J Natl Compr Canc Netw 2016; 14: 1286-1312.
[89] Casak SJ, Fashoyin-Aje I, Lemery SJ, Zhang L, Jin R, Li H, Zhao L, Zhao H, Zhang H, Chen H, He K, Dougherty M, Novak R, Kennett S, Khasar S, Helms W, Keegan P and Pazdur R. FDA approval summary: ramucirumab for gastric cancer. Clin Cancer Res 2015; 21: 3372-3376.
[90] Brahmer JR, Tykodi SS, Chow LQ, Hwu WJ, Topalian SL, Hwu P, Drake CG, Camacho LH, Kauh J, Odunsi K, Pitot HC, Hamid O, Bhatia S, Martins R, Eaton K, Chen S, Salay TM, Alaparthy S, Grosso JF, Korman AJ, Parker SM, Agrawal S, Goldberg SM, Pardoll DM, Gupta A and Wigginton JM. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. N Engl J Med 2012; 366: 2455-2465.
[91] Muro K, Chung HC, Shankaran V, Geva R, Catenacci D, Gupta S, Eder JP, Golan T, Le DT, Burtness B, McRee AJ, Lin CC, Pathiraja K, Lunceford J, Emancipator K, Juco J, Koshiji M and Bang YJ. Pembrolizumab for patients with PD-L1-positive advanced gastric cancer (KEYNOTE-012): a multicentre, open-label, phase 1b trial. Lancet Oncol 2016; 17: 717-726.
[92] Ma C, Patel K, Singhi AD, Ren B, Zhu B, Shaikh F and Sun W. Programmed death-ligand 1 expression is common in gastric cancer associated with epstein-barr virus or microsatellite instability. Am J Surg Pathol 2016; 40: 1496-1506.
[93] Jin Z and Yoon HH. The promise of PD-1 inhibitors in gastro-esophageal cancers: microsatellite instability vs. PD-L1. J Gastrointest Oncol 2016; 7: 771-788.
[94] Root A, Cao W, Li B, LaPan P, Meade C, Sanford J, Jin M, O'Sullivan C, Cummins E, Lambert M, Sheehan A, Ma W, Gatto S, Kerns K, Lam K, D'Antona A, Zhu L, Brady W, Benard S, King A, He T, Racie L, Arai M, Barrett D, Stochaj W, La-

Vallie E, Apgar J, Svenson K, Mosyak L, Yang Y, Chichili G, Liu L, Li H, Burke S, Johnson S, Alderson R, Finlay W, Lin L, Olland S, Somers W, Bonvini E, Gerber H-P, May C, Moore P, Tchistiakova L and Bloom L. Development of PF-06671008, a highly potent Anti-P-cadherin/Anti-CD3 bispecific DART molecule with extended half-life for the treatment of cancer. Antibodies 2016; 5: 6.

[95] Guilford P, Hopkins J, Harraway J, McLeod M, McLeod N, Harawira P, Taite H, Scoular R, Miller A and Reeve AE. E-cadherin germline mutations in familial gastric cancer. Nature 1998; 392: 402-405.

[96] Majewski IJ, Kluijt I, Cats A, Scerri TS, de Jong D, Kluin RJ, Hansford S, Hogervorst FB, Bosma AJ, Hofland I, Winter M, Huntsman D, Jonkers J, Bahlo M and Bernards R. An alpha-E-catenin (CTNNA1) mutation in hereditary diffuse gastric cancer. J Pathol 2013; 229: 621-629.

[97] Liang H and Kim YH. Identifying molecular drivers of gastric cancer through next-generation sequencing. Cancer Lett 2013; 340: 241-246.

[98] Kamata T, Sunami K, Yoshida A, Shiraishi K, Furuta K, Shimada Y, Katai H, Watanabe S, Asamura H, Kohno T and Tsuta K. Frequent BRAF or EGFR mutations in ciliated muconodular papillary tumors of the lung. J Thorac Oncol 2016; 11: 261-265.

[99] Min BH, Hwang J, Kim NK, Park G, Kang SY, Ahn S, Ahn S, Ha SY, Lee YK, Kushima R, Van Vrancken M, Kim MJ, Park C, Park HY, Chae J, Jang SS, Kim SJ, Kim YH, Kim JI and Kim KM. Dysregulated Wnt signalling and recurrent mutations of the tumour suppressor RNF43 in early gastric carcinogenesis. J Pathol 2016; 240: 304-314.

[100] Chan TH, Qamra A, Tan KT, Guo J, Yang H, Qi L, Lin JS, Ng VH, Song Y, Hong H, Tay ST, Liu Y, Lee J, Rha SY, Zhu F, So JB, Teh BT, Yeoh KG, Rozen S, Tenen DG, Tan P and Chen L. ADAR-mediated RNA editing predicts progression and prognosis of gastric cancer. Gastroenterology 2016; 151: 637-650, e610.

[101] Tsai KW, Chang B, Pan CT, Lin WC, Chen TW and Li SC. Evaluation and application of the strand-specific protocol for next-generation sequencing. Biomed Res Int 2015; 2015: 182389.