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Biomarker Discovery

Multi-omic Solutions for Pharmaceutical Research

Biomarkers are indicators of disease occurrence and progression. Biomarkers can be used to measure and evaluate normal biological and pathogenic processes, or pharmacologic responses to a therapeutic intervention, and in some cases they may serve as potential drug targets.

Biomarkers can be specific cells, molecules, genes, gene products, enzymes, or hormones. Complex organ functions or general characteristic changes in biological structures can also serve as biomarkers. There are three major classified types of biomarkers according to their application in disease detection: screening, diagnostic and prognostic biomarkers. Screening biomarkers are used to predict the potential occurrence of a disease in asymptomatic patients. Diagnostic biomarkers are used to make predictions on patients suspected of a certain disease. Prognostic biomarkers are applied to predict the outcome of a patient suffering from a specific disease.

Biomarkers play a major role in medicinal biology. Advances in this area have traditionally been driven by the intersection of the medical and biological sciences. Nevertheless, it is evident that current and future progress also relies on the combination of skills and resources originating from the physical and computational sciences and engineering. In particular, with advances of high-throughput technologies and bioinformatics, there begins a new era of drug research and development.

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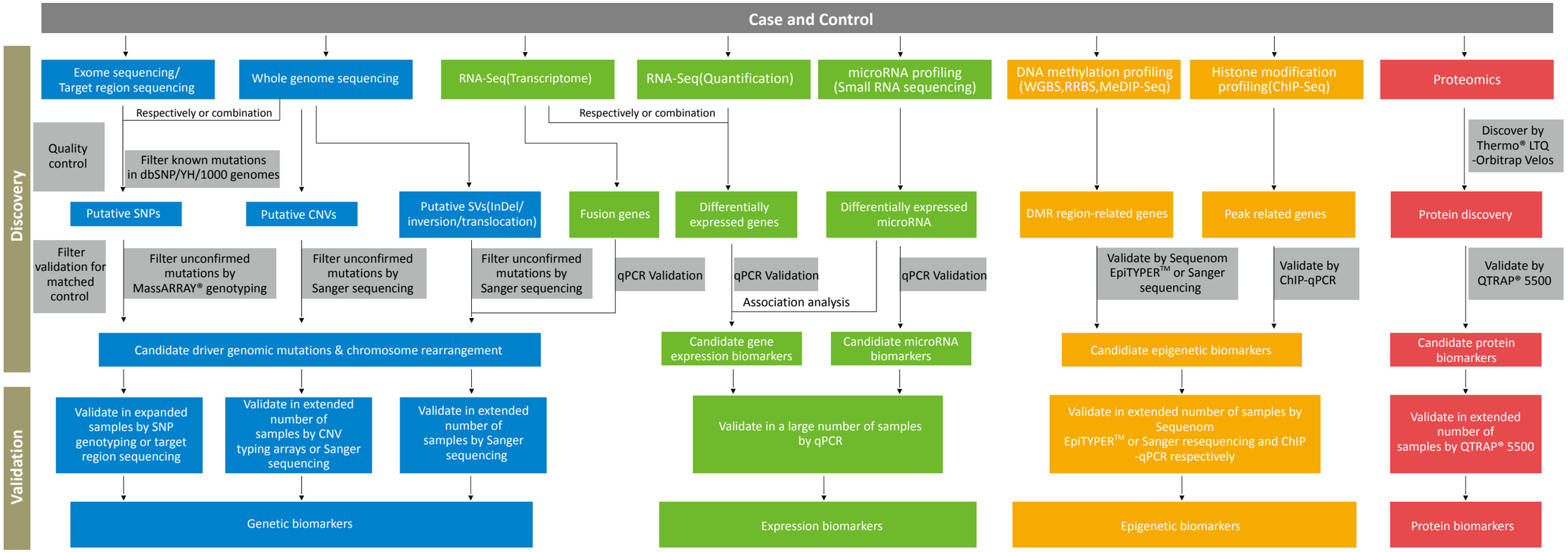
Overview of Multi-omic Approaches Applied in Biomarker Discovery



Solutions for Genetic Biomarkers

Potential genomic variation-based biomarkers can be initially predicted by finding strong genotype-phenotype associations in a specific population sample. There are three important DNA variations: SNP (single nucleotide polymorphism), SV (structural variation) and CNV (gene copy number variation).

Solutions for SNP Biomarkers

SNPs are sequence variants at single nucleotide level. SNPs can affect gene expression and protein function by altering not only protein-coding regions, but also non-coding regions[1].

SNPs in the DNA sequences of humans can affect the development of human diseases and respond to pathogens, chemicals, drugs, vaccines, and other agents. SNPs are also thought to be key enablers in realizing the concept of personalized medicine.

Over the past few years, increasingly extensive systematic sequencing of cancer genomes has identified several new mutated cancer genes. Examples include *BRAF, PIK3CA, FGFR2, JAK2 and IDH1*[2-6], and searches for inhibitors of the proteins encoded by many of these mutated genes are in progress.

Solutions

Solution 1: Whole genome sequencing (WGS) & Genotyping validation/ Target region sequencing

- Achieve the most comprehensive detection and analysis of genomic variations in protein-coding and non-coding regions
- Suitable for pre-discovery stage

Solution 2: Exome sequencing / Target region sequencing & Genotyping validation

- Focus on detection and analysis of genomic variations in exon regions or other selected regions
- Efficient strategy to selectively sequence with deep sequencing
- Cost-effective

Sample Selection

Solution 1

- Discovery stage: 100 or more paired cases and controls from random samples are suggested
- Validation stage: 500 or more patient blood or fresh-frozen / FFPE samples

Solution 2

- Sample selection for solution 2 is demonstrated in Table 1.

Table 1 Sample selection options of exome sequencing

Sample Size (Cases /Control)	Option 1	Option 2	Option 3	Option 4
Stage 1-Exome sequencing	100 /100	300 /300	500 /500	1000 /1000
Stage 2-Genotyping validation	1000 /1000	2000 /2000	4000 /4000	10000 /10000
Research purpose	≥ 8.98% (MAF≥0.02)	≥54.38% (MAF≥0.02)	≥77.20% (MAF≥0.02)	≥96.42% (MAF≥0.02)
Discovery of disease relative SNP	≥ 43.53% (MAF≥0.05)	≥90.76% (MAF≥0.05)	≥98.32% (MAF≥0.05)	≥99.96% (MAF≥0.05)

Note: The percentage of disease related SNPs and MAF values is calculated by LRT (Likelihood Ratio Test)[7].
MAF: Minor Allele Frequency

Research Workflow

Figure 1 and Figure 2 show the workflow of SNPs biomarker solutions.

Figure 1 General workflow of SNP biomarker solutions

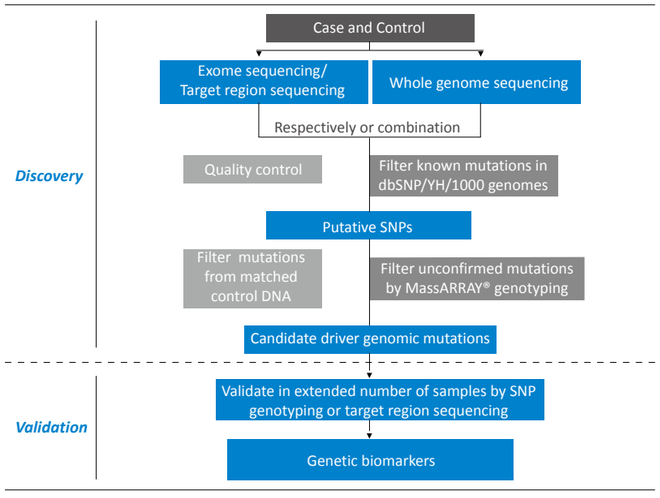


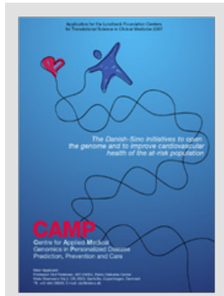
Figure 2 Detailed workflow of SNP biomarker screening

Expected Results

- Identification and validation of patient stratification biomarkers in clinical samples
- Discovery of biomarkers related to disease or drug activity
- Personalized medicine by SNPs biomarkers

Case Study

The Sino-Danish Project is held by LUCAMP gathering Hagedorn Research Institute, Steno Diabetes Center, Danish Universities and BGI, mainly focus on metabolic disorders. The aim is to find the new common and rare genetic variations and test the frequency differences between variations in patients and healthy individuals.



Objectives

- Identify novel genetic variation, both common and rare associated with disease state
- Identify differences in the allele frequency of genetic variations between disease-affected and control groups

Samples

- 1000 patients with gender-defined visceral obesity, type 2 diabetes and essential hypertension
- 1000 age- and gender-matched controls who are glucose-tolerant, lean and normotensive

Primary Results

Low frequency genetic variants were found to be more likely non-synonymous changes (Figure 3)[8].

These results support the theory that the heritable variation affecting fitness is majorly caused by low-frequency mutations, which are often overlooked in studies based on genotyping rather than resequencing. Furthermore, it is demonstrated that next-generation sequencing is an important tool to find the 'missing heritability' of complex traits including cardiovascular diseases.

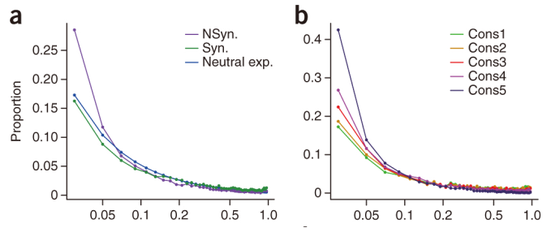


Figure 3 Comparison of site frequency spectra for SNPs in different annotation categories with functional consideration.

Note: Unfolded site frequency spectra (SFS) for non-synonymous (Nsyn) and synonymous (Syn) SNPs compared to the neutral
Non-synonymous SFS for sites having increasing conservation scores, with Cons5 being the most conserved category

Solutions for Structural Variation Biomarkers

Structural variations (SVs) are the variations in structure of chromosome, including deletions, duplications, insertions, inversions and translocations. Typically the lengths of SVs are in the range of 1 Kb to 3 Mb, which are larger than SNPs and smaller than large-scale chromosome abnormality[9] The importance of SVs to human health and common genetic disease has become increasingly apparent. SVs have been implicated to overlap disease-causing genes, alter gene-expression levels and contribute to disease susceptibilities such as in HIV infection, systemic autoimmunity, genomic disorders such as Williams-Beuren syndrome[10] and velo-cardio-facial syndrome[11]. The full extent of structural variation is important for understanding phenotypic variation and genetic disease in humans.

With the completions of human genome sequencing, the operational resolution of SVs has been widened to include much smaller events[14]. In addition to SVs at genomic level, gene fusion at transcriptomic level is also an important factor which regulates and controls gene expression in human organisms.

Solutions

BGI can provide the following biomarker solution for SV, which is combination of WGS, RNA-Seq (Transcriptome) and Sanger re-sequencing.

- Sample Selections

Discovery Stage: 100 or more paired cases and controls for random samples are suggested (30 or more for family samples)

Validation Stage: 500 or more fresh-frozen or FFPE samples

- Research Workflow

Whole genome sequencing is used to find the disease-related SVs and RNA-Seq (Transcriptome) is combined to validate the partial results through identifying the fusion gene.

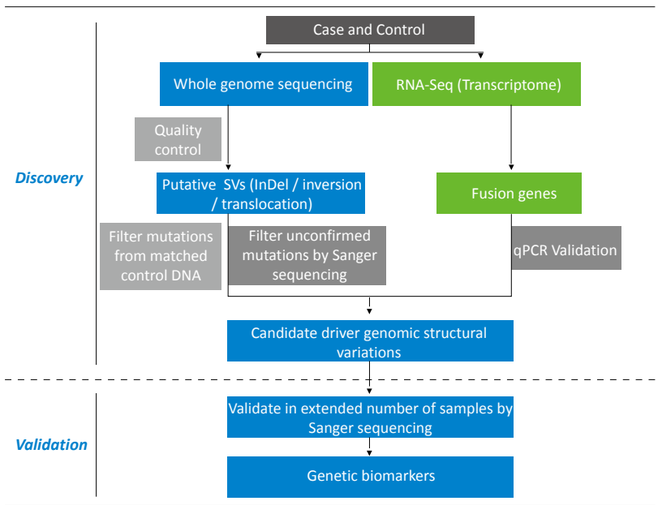


Figure 4 Detailed workflow of SV biomarker screening

Solutions for Copy Number Variation Biomarkers

Copy Number Variations (CNVs) are genomic variations in which blocks of DNA are missing or duplicated. Different potentially-significant associations between CNVs and disease have been reported in Chron's disease, schizophrenia, autism, several cancers, and retinoblastoma. To understand the connection between CNVs and disease, it requires to be estimated that chromosomal locations and numbers of CNVs in both healthy and diseased groups[6].

Solutions

- Sample Selection

Discovery Stage: 100 or more paired cases and controls are suggested

Validation Stage: 500 or more fresh-frozen or FFPE samples

- Research Workflow

Based on read depth, CNVs are identified by the program CNV detection (such as CNV detector).

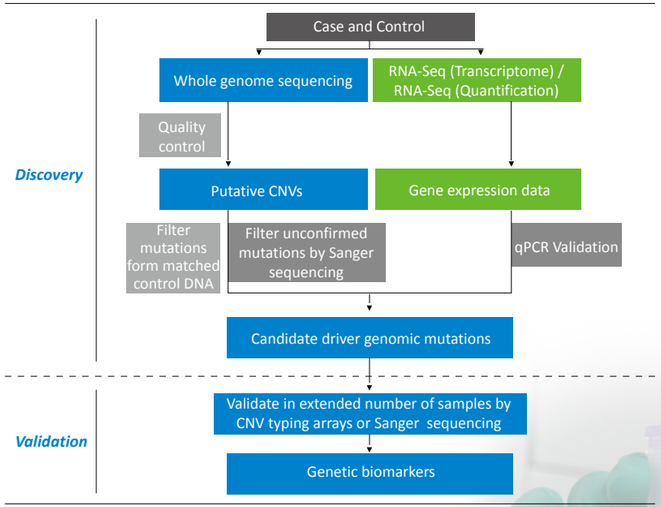


Figure 5 Detailed workflow of CNV biomarker screening

Solutions for Gene Expression Biomarkers

Differentially expressed genes (DEGs) have been used as the starting point of different biomarker discoveries. Van't Veer, *et al.* (2002) reported their research on the discovery of biomarkers based on gene expression profiling for supporting the prediction of clinical outcomes[13]. Moreover, gene-expression biomarkers have been used for diagnosis of sub-classes of cancers and prognosis in breast and lung cancers. The combination of microarrays and genomic variation analysis through sequencing of diverse genes has enabled a more detailed characterization of individual cancers[14]. More recently, the application of whole-blood or plasma-based gene expression profiling has been proposed as a novel alternative for biomarker discovery[15-16].

Solutions

- **Solution 1**: RNA-Seq (Quantification) & Proteomics
- **Solution 2**: RNA-Seq (Quantification) & Epigenomics & Small RNA sequencing

Solutions

Solution 1: RNA-Seq (Quantification) & Proteomics

BGI performs RNA-Seq (Quantification) to detect DEGs and combines proteomic data to confirm candidate biomarkers. These approaches can provide the information regarding genes and proteins that are expressed under certain conditions.

- Sample Selection

RNA-Seq (Quantification) Samples: 500 or more paired cases and controls are suggested.

Proteomic Samples: The same samples as for RNA-Seq (Quantification).

- Research Workflow

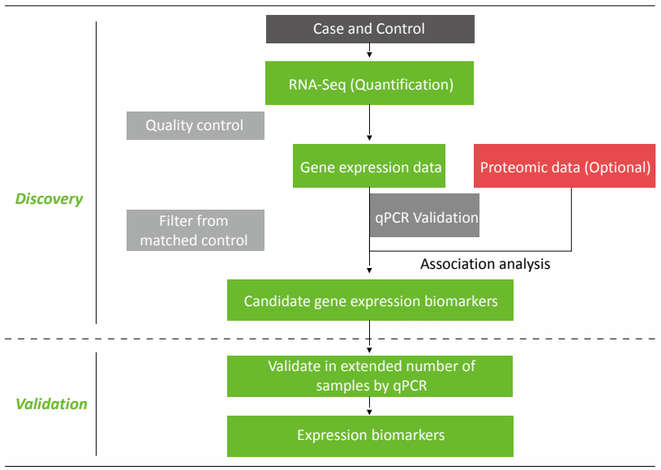


Figure 6 Workflow of gene expression biomarkers screening associated with proteomic data

- Expected Results

Integration of gene expression and proteomic data to acquire the high quality candidate biomarkers

Solution 2: RNA-Seq (Quantification) & Epigenomics & Small RNA sequencing

DNA methylation, histone modification, non-coding RNA regulations and other so called “epigenomic” changes can regulate gene expression[17-18]. After RNA-Seq (Quantification), DNA methylome, ChIP-Seq or small RNA sequencing could be applied to predict regulators with regulatory motif instances affected and target genes with expression altered, thereby suggesting specific mechanistic hypotheses for how disease-associated genotypes lead to the observed disease phenotypes[19], which is another way to confirm gene expression biomarkers found by RNA-Seq.

- Sample Selection

RNA-Seq (Quantification) Samples: 500 or more paired cases and controls are suggested.

Epigenomics Samples: The same samples as for RNA-Seq (Quantification).

Small RNA Sequencing Samples: The same samples as for RNA-Seq (Quantification).

- Research Workflow

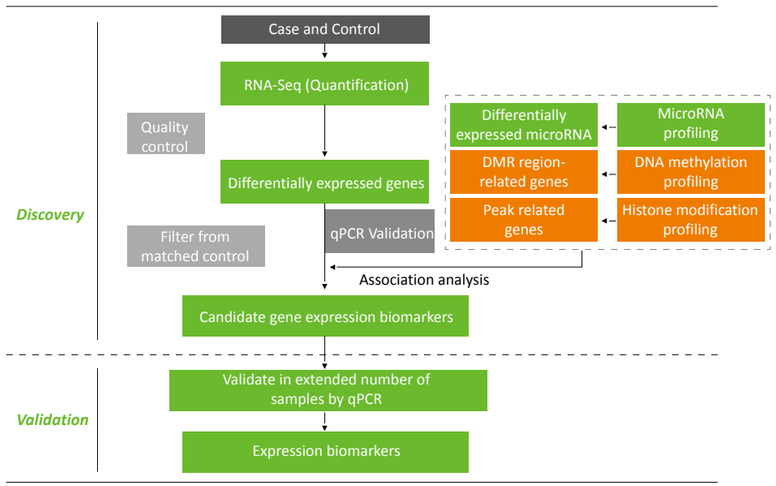


Figure 7 Workflow of gene expression biomarkers screening associated with epigenomic changes and microRNA profiling

- Expected Results

- Identification and validation of gene expression biomarkers in clinical samples
- Discovery of biomarkers related to disease or drug activity
- Personalized medicine by gene expression biomarkers

Solutions for MicroRNA Biomarkers

MicroRNA is a small non-coding RNA with length about 22 nt, which plays an important role in gene posttranscriptional regulation, such as transcript degradation and sequestering, translational suppression. It is evident that microRNA associates with many diseases, such as cancer, infection, cardiovascular diseases, *etc.* Patterson, *et al.* revealed *miR-483* as a marker of adrenocortical tumors[20]. Asaga, *et al.* detected circulating *miR-21* to diagnose and prognose potential in breast cancer[21]. The unique expression patterns of serum microRNAs under a specific disease condition imply a great potential of serum microRNA profiling as the fingerprint for diseases[22-23]. The diagnosis, prognosis, and treatment of these diseases are important potential clinical applications of microRNA profiling[24-27].

Nowadays, there are number of ways to screen the microRNA profiling, such as qPCR, microarray and small RNA sequencing. Small RNA sequencing is based on NGS with the advantages of high throughput and single nucleotide resolution, which is the best choice for microRNA studies.

Solutions

BGI suggests the following solutions for microRNA biomarkers discovery. The workflow is shown as in Figure 8.

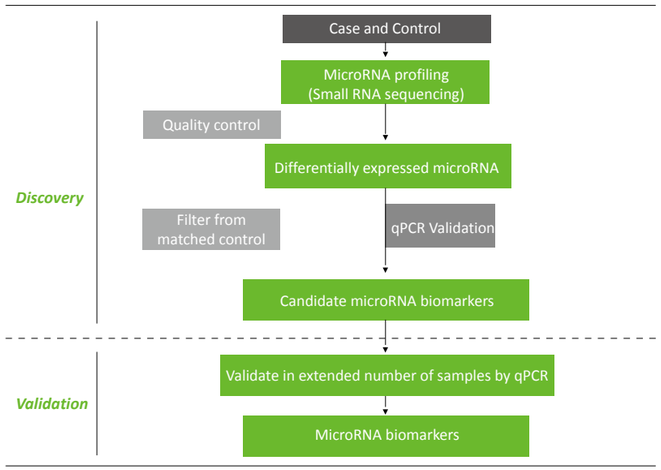


Figure 8 Solutions of screening microRNA biomarkers

- BGI Has Experience with The Following Sample Types

Tissue, cell line, serum, *etc.*

- Expected Results

Detect microRNA molecular biomarkers

Case Study

Shen, *et al.* revealed that the four-microRNAs signature from serum may serve as a noninvasive predictor for the overall survival of NSCLC (Non–Small-Cell Lung Cancer)[23]. Genome-wide serum microRNA expression analysis was used to investigate the role of serum microRNA in predicting prognosis of NSCLC. There are distinct differences in levels of serum microRNAs between longer and shorter survival groups, which have 30 patients respectively, and qPCR assays was used to validate in serum microRNAs of 243 patients (Figure 9).

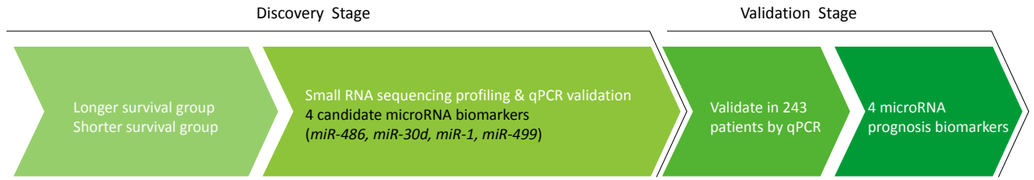
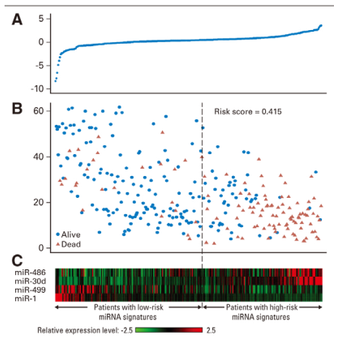


Figure 9 Workflow of microRNA screening

Note: Longer survival: alive and mean survival time, 49.54 months
Shorter survival: dead and mean survival time, 9.54 months

The distribution of serum microRNA expression levels, patient risk scores, and survival time of the all-combined 303 patients were shown in Figure 10. Patients with high-risk scores tended to have high-risk microRNAs, whereas patients with low-risk scores tended to have more protective microRNAs. Patients with high-risk scores died sooner than those with low-risk scores.

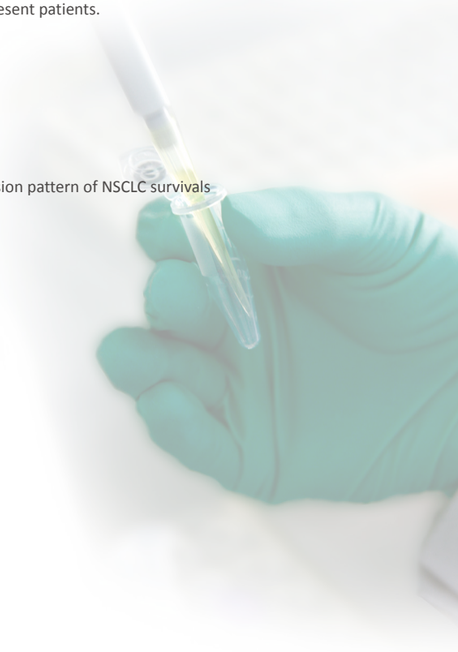


Note:(A) Risk-score distribution
Risk-score = (0.969 X expression level of *miR-486*)+ (0.973 Xexpression level of *miR-30d*) + (-0.650 X expression level of *miR-1*) + (-0.815 X expression level of *miR-499*).

(B) Patients' survival status and time (months)

(C) Color-gram of microRNA expression profiles of patients with NSCLC, rows represent microRNAs, and columns represent patients.

Figure 10 The correlation among risk score, survival time and microRNA expression pattern of NSCLC survivals



Solutions for Epigenetic Biomarkers

Epigenetics involves the study of changes in the regulation of gene activity and expression independent from the DNA sequence, which refers to a more global analysis of epigenetic changes across the entire genome.

The best-known epigenetic marker is DNA methylation, a process in which enzymes attach methyl groups onto genes and block their activity. Other so-called epigenetic changes such as histone modification might be as important as genetic mutations in causing cancer[17].

So far, many epigenetic biomarkers are used in the diagnosis of cancer, such as *m*SEPT9 for colorectal cancer, *m*SHOX2 for lung cancer, *m*PITX2 and *m*GSTP1 for prostate cancer, *etc*[28-31]. Besides, DNA demethylating drugs (DNMT-i) in low doses have clinical activity against some tumors. Two such agents, 5-azacytidine (Vidaza) and 5-aza-2'- deoxycytidine (decitabine), have been approved as treatments for the myelodysplastic syndrome and leukemia. And the first drug of histone deacetylase inhibitors (HDAC-i), suberoylanilide hydroxamic acid (vorinostat), has been approved by the FDA for the treatment of cutaneous T-cell lymphoma.

Solutions

BGI can offer the whole solution to study epigenomics (Figure 11).

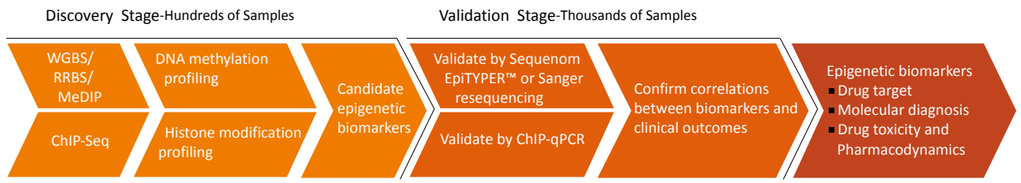


Figure 11 Workflow of screening epigenetic biomarkers

- BGI Has Experience with The Following Sample Types

Tissue, cell line and blood samples

- Expected Results

- Identification and validation of epigenetic biomarkers in clinical samples
- Discovery of epigenetic biomarkers related to disease or drug activity
- Personalized medicine by epigenetic biomarkers

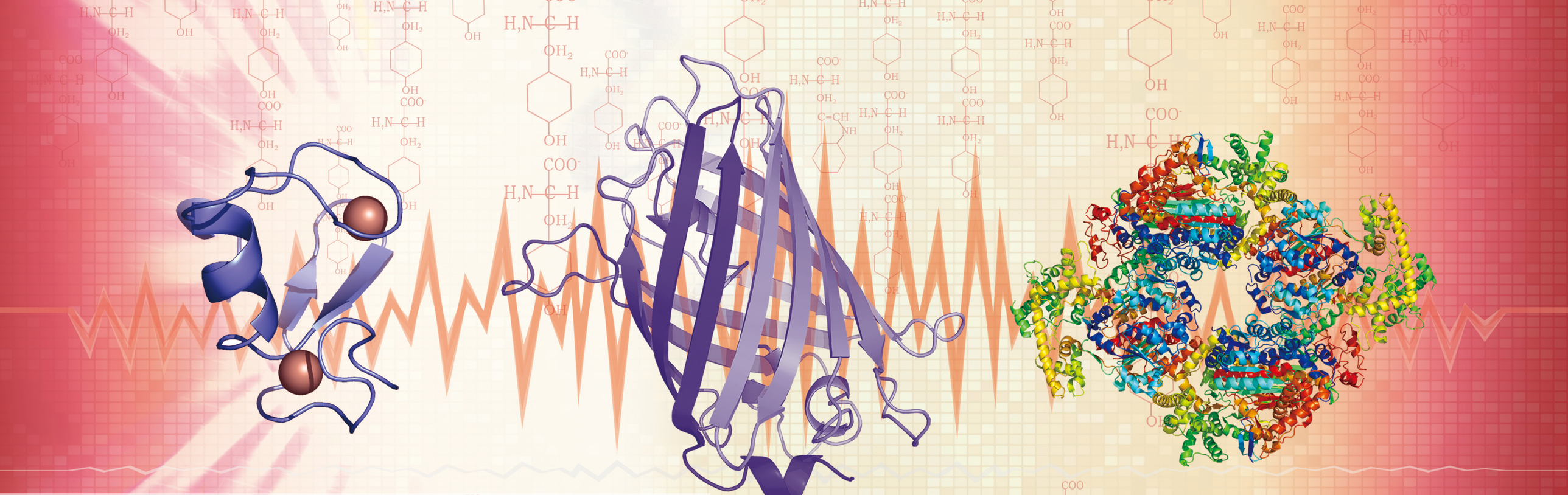
Epigenetic Aberrations in Cancer

DNA methylation techniques permit the sensitive and quantitative detection of hypermethylated tumor-suppressor genes in various types of biologic fluids and biopsy specimens. The establishment of DNA methylation and histone-modification profiles of the primary tumor specimen itself might be a valuable tool in determining the prognosis and predicting the patient's response to therapies. The epigenetic aberrations among different tumor types are shown in Table 2. These epigenetic changes which can modify gene expression gave us clues for human epigenome projects and epigenetic therapies[32].

Table 2 Epigenetic Aberrations among Different Tumor Types[32]

Type of Cancer	Epigenetic Disruption
Colon cancer	CpG-island hypermethylation (*hMLH1*, $p16^{INK4a}$, $p14^{ARF}$, *RARB2*, *SFRP1*, and *WRN*), hypermethylation of microRNAs (*miR-124a*), global genomic hypomethylation, loss of imprinting of *IGF2*, mutations of histone modifiers (*EP300* and *HDAC2*), diminished monoacetylated and trimethylated forms of histone H4
Breast cancer	CpG-island hypermethylation (*BRCA1*, E-cadherin, *TMS1*, and estrogen receptor), global genomic hypomethylation
Lung cancer	CpG-island hypermethylation ($p16^{INK4a}$, *DAPK*, and *RASSF1A*), global genomic hypomethylation, genomic deletions of *CBP* and the chromatin-remodeling factor *BRG1*
Glioma	CpG-island hypermethylation (DNA-repair enzyme *MGMT, EMP3*, and *THBS1*)
Leukemia	CpG-island hypermethylation ($p15^{INK4b}$, *EXT1*, and *ID4*), translocations of histone modifiers (*CBP, MOZ, MORF, MLL1, MLL3*, and *NSD1*)
Lymphoma	CpG-island hypermethylation ($p16^{INK4a}$, *p73*, and DNA-repair enzyme *MGMT*), diminished monoacetylated and trimethylated forms of histone H4
Bladder cancer	CpG-island hypermethylation ($p16^{INK4a}$ and *TPEF/HPP1*), hypermethylation of microRNAs (*miR-127*), global genomic hypomethylation
Kidney cancer	CpG-island hypermethylation (*VHL*), loss of imprinting of *IGF2*, global genomic hypomethylation
Prostate cancer	CpG-island hypermethylation (*GSTP1*), gene amplification of polycomb histone methyltransferase *EZH2*, aberrant modification pattern of histones H3 and H4
Esophageal cancer	CpG-island hypermethylation ($p16^{INK4b}$ and $p14^{ARF}$), gene amplification of histone demethylase *JMJD2C/GASC1*
Stomach cancer	CpG-island hypermethylation (*hMLH1* and $p14^{ARF}$)
Liver cancer	CpG-island hypermethylation (*SOCS1* and *GSTP1*), global genomic hypomethylation
Ovarian cancer	CpG-island hypermethylation (*BRCA1*)

Note: *BRCA1* denotes breast-cancer susceptibility gene 1, *BRG1* BRM/SWI2-related gene 1, *CBP* cyclic AMP response- element binding protein (CREB)–binding protein, *DAPK* death-associated protein kinase, *EMP3* epithelial membrane protein 3, *EP300* E1A binding protein p300, *EXT1* exostosin 1, *EZH2* enhancer of zeste drosophila homologue 2, *GSTP1* glutathione S-transferase 1, *HDAC2* histone deacetylase 2, *hMLH1* homologue of *MutL* Escherichia coli, *ID4* inhibitor of DNA binding 4, *IGF2* insulin-like growth factor 2, *JMJD2C/GASC1* Jumonji domain-containing protein 2C, *MGMT* O6-methyl-guanine DNA methyltransferase, *MLL1* mixed-lineage leukemia 1, *MLL3* mixed-lineage leukemia 3, *MORF* monocytic leukemia zinc finger protein related factor, *MOZ* monocytic leukemia zinc finger, *NSD1* nuclear receptor binding SET-domain protein 1, *RARß2* retinoic acid receptor β2, *RASSF1A* ras association domain family protein 1, *SFRP1* secreted frizzled-related protein 1, *SOCS1* suppressor of cytokine signaling 1, *THBS1* thrombospondin 1, *TMS1* target of methyl-ation-induced silencing 1, *TPEF/HPP1* hyperplastic polyposis gene 1, *VHL* von Hippel Lindau disease, and *WRN* Werner's syndrome.



Solutions for Protein Biomarkers

Solutions for Protein Biomarkers

Recent advances in the SRM/MRM technology show the potential to bridge the gap between the generation of candidate lists and their verification[33]. The strength of these technologies is the ability to develop sensitive and selective assays for proteins in a more cost-effective and time-saving manner compared to standard ELISA assays. BGI's proteomics platform, integrating the most advanced mass spectrometry technologies, provide sensitive measures of protein changes occurring in *vivo*. BGI protein biomarker services can provide the whole research programs of protein biomarker discovery, verification and validation.

Solutions

BGI has built up a range of LC-MS proteomics platforms including the most advanced mass spectrometry such as LTQ-Orbitrap Velos from Thermo, ultrafleXtreme™ and maXis™ from Bruker, QTRAP® 5500 from AB SCIEX. Besides, BGI can perform professional bioinformatics analysis.

The comprehensive solutions for protein biomarker discovery, verification and validation are shown in Figure 12.

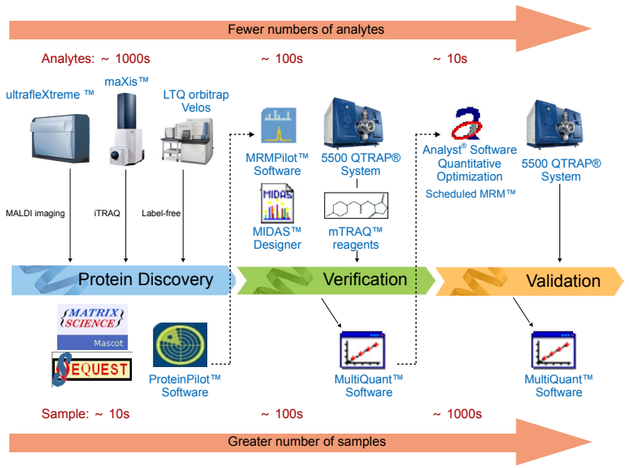


Figure 12 Whole Solutions for Protein Biomarker in BGI

- Our Services for Protein Biomarkers

  - Biomarker discovery, verification and validation
  - Protein and peptide target discovery and identification
  - Identification of regulated proteins and protein species
  - Pharmaco proteomics
  - Identification of vaccine candidates
  - Immuo proteomics
  - Plasma Proteomics
  - Phospho proteomics
  - Membrane proteomics

- BGI Has Experience with The Following Sample Types

Human plasma, biofluids, cell lysate, tissues, animal model plasma, proximal fluids, *etc*.

Case Study

We reported a new set of data for screening the biomarkers from the gastric tissues on the basis of the proteomic strategy developed in our laboratory. Comparative analysis of the protein profiles from primary gastric tumors and their adjacent regions by MALDI-TOF/TOF MS, MAWBP was identified to could be a new protein candidate involved in gastric cancer (GC)[34].

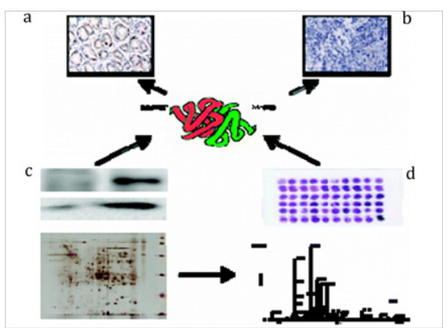


Figure 13 The overview of protein biomarker discovery

Note: The gastric proteins were well-resolved by 2-DE (Figure 13 c), and then the GC-associated proteins were identified by MALDI-TOF/TOF MS following image analysis(Figure 13 d), and also expanded a systematical examination immunohistochemical staining to validate the putative biomarker in a large number of the GC cases(Figure 13 a and b).

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