Cancer gene signatures in risk stratification: use in personalized medicine

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Cancer is a complex disease which arises due to a series of genetic changes related to cell division and growth control. Cancer remains the second leading cause of death in humans next to heart diseases. As a testimony to our progress in understanding the biology of cancer and developments in cancer diagnosis and treatment methods, the overall median survival time of all cancers has increased six fold – one year to six years – during the last four decades. However, while the median survival time has increased dramatically for some cancers like breast and colon, there has been only little change for other cancers like pancreas and brain. Further, not all patients having a single type of tumour respond to the standard treatment. The differential response is due to genetic heterogeneity which exists not only between tumours, which is called intertumour heterogeneity, but also within individual tumours, which is called intratumoural heterogeneity. Thus it becomes essential to personalize the cancer treatment based on a specific genetic change in a given tumour. It is also possible to stratify cancer patients into low- and high-risk groups based on expression changes or alterations in a group of genes – gene signatures and choose a more suitable mode of therapy. It is now possible that each tumour can be analysed using various high-throughput methods like gene expression profiling and next-generation sequencing to identify its unique fingerprint based on which a personalized or tailor-made therapy can be developed. Here, we review the important progress made in the recent years towards personalizing cancer treatment with the use of gene signatures.

Keywords: Biomarker, cancer, molecular signature, personalized medicine.

Introduction

ONE of the most familiar and dreadful diseases at present is cancer. Though, in most simplified terms, it is a result of mere imbalance in cell growth and death, it has left scientists raking their brains for the possible causes and cures. Years of study has associated many internal factors (inherited mutations, hormonal changes, change in immune conditions and metabolic problems) and external factors (tobacco, radiation, different chemical carcinogens and infectious organisms) with cancer[1–3]. These factors may contribute to the accumulation of different abnormalities by changing genetic or epigenetic composition of the genome, which may lead to acquisition of many important traits by cancerous cells, including losing their control on division, migration and invasion and even resistance to radio and chemotherapy.

Malignancies which are frequently caused by external factors like tobacco consumption in lung cancer, can be prevented by eliminating these exposures. Similarly, the occurrence of certain other cancers can be predicted by early detection of inherited mutations frequently associated with a particular type of cancer. Early diagnosis and improved treatment protocol have contributed to the improved survival of cancer patients, but still around 7.6 million (around 13% of all deaths) people died because of cancer in 2008 (ref. 4). Cancer causes more deaths than AIDS, tuberculosis and malaria combined. Based on information provided by the World Health Organization (WHO), lung, stomach, liver, colon and breast cancer cause the most disease-related deaths[5].

Current methods of cancer treatment and their limitations

The current cancer treatment regime involves surgery, radiotherapy and chemotherapy, depending upon the location, type and stage of tumour. Removal of cancer tissue by surgery is the most common practice in cancer treatment. Surgical resection involves maximum possible removal of cancerous tissue. In many cancers, surgery is followed by radiotherapy and or chemotherapy. Radiotherapy is given in the form of ionizing radiation, which works by damaging the DNA leading to cell death. In addition to radiotherapy, high-grade tumours are also treated with different types of chemotherapy, which includes treatment with single or multiple drugs. For example, breast cancer treatment includes adriamycin and taxol[6], while for glioblastoma, the most aggressive brain cancer, the treatment includes temozolomide treatment[7].

A new emerging type of cancer treatment is targeted therapy in which drugs or other reagents specifically target and kill cancer cells with little or no damage to normal cells. The target is usually a protein which is

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essential for cancer growth and survival. A number of targeted therapies are being used for various cancers[2,8,9]. With the development of new technologies, there have been many success stories in other aspects of cancer therapy in recent years. The advancements in surgical techniques, modern high-voltage irradiation methods and newer chemotherapeutic molecules have contributed in a substantial increase in the survival time in many cancers. However, even with best possible treatments available today, all patients having a single type of tumour do not respond to the therapy equally. This difference in response to therapy by different patients may be attributed to the genetic heterogeneity of the tumours[10]. In simple words, all cancers belonging to a particular type are not the same as they have different genetic and epigenetic make-up and thus they respond differently towards certain therapies and may require alternate treatments. As tumour heterogeneity arises due to varying genetic alterations between tumours, it is now possible by the use of high-throughput techniques like microarray and next-generation sequencing (NGS) to develop robust diagnostic, predictive and prognostic markers as well as identify specific targets to choose the right kind of therapy. Using these, personalized therapy can be designed for each patient. In this review, we will mainly focus on the current status of gene signatures in personalizing cancer treatment. There are many excellent published reviews on targeted therapies in cancer treatment[2,8,9].

Predictive and prognostic markers

Biomarkers are increasingly used in the management of cancer. Broadly, for the personalized cancer therapy biomarkers can be divided into two types – predictive and prognostic. Prognostic biomarkers are defined as 'the markers that can predict the outcome of a cancer disease in an untreated patient'. These markers are helpful for identifying the patients who are at high risk and therefore can be considered for aggressive therapy[11].

In contrast to prognostic markers, predictive biomarkers are defined as 'the markers which can be used to identify subclass of patients who are most likely to respond to a given therapy'. These markers may help in selecting the proper therapy for individual patients[11]. Prognostic markers (also called prognostic variables or factors) are important factors in the management of cancer. These markers help in stratification of patients into different risk groups and therefore help in management of the treatment protocol. These markers can be divided into two types – single factor-based markers and gene signatures.

Single factor-based markers are based on the behaviour of a single factor across tumours. For example, estrogen receptor expression level is a prognostic marker in breast cancer[12]. These markers are easy to use as only one factor status has to be determined, but may suffer with less reliability. In contrast to single factor-based markers, a molecular signature is the group of molecular factors whose combined pattern can predict the outcome[13]. These genes are tightly co-regulated and may or may not function as individual markers. Molecular signatures are not as user-friendly as the single factor-based markers, but have high reliability and robustness. These gene signatures are based on microarray technology, which provides an ideal tool for comprehensive molecular and genetic profiling of cancer.

Prognostic molecular signatures and risk stratification

The molecular signature for prognosis is a useful tool to classify tumours into different risk groups which would help in choosing the right treatment option. There are many prognostic molecular signatures under different stages of development and validation, with some are already in use for cancer treatment (Table 1). Here, we will discuss various signatures which are being used in clinics as well as at various stages of validation.

Breast cancer gene signatures

Breast cancer is one of the cancers in which molecular signatures greatly help in deciding the treatment protocol. Breast cancer is the major cause of disease-related death in developed countries. Many pathological factors and clinical features, for example, age, tumour size, menopausal status, grade of tumour, lymph node metastasis status, ERBB2 receptor status and estrogen receptor (ER) status have been shown to have prognostic value in breast cancer patients[12]. Although these markers give valuable information about patient's outcome, they have only limited ability in prediction. This paved the way to the discovery of many prognostic gene signatures in breast cancer. Numerous studies that followed contributed in making breast cancer to be the leading example for which prognostic gene signatures are already in use. The currently used prognostic signatures in breast cancer are described below.

MammaPrint: This is a trade name of 70-gene prognostic signature of breast cancer. This signature was first developed by The Netherlands Cancer Institute in Amsterdam (NKI) using Agilent microarray platform. This signature was derived from 78 systemically untreated lymph node-negative breast cancers of patients in the age group less than 55 years. Out of 78 patients, 44 were metastasis free and 34 patients had distant metastasis within 5 years. The signature was identified using three-step supervized classification method and was then validated by the same group on a larger dataset of 295

Table 1. Cancer gene signatures in different stages of development

Signature	Use	No. of genes	Platform	Independent validation	Reference
Gastrointestinal cancer					
6-gene signature	Likelihood of relapse	6	Illumina	No	26
Colo guide pro	Prognosis	7	Affymetrix GeneChip	Yes	27
5-gene expression signature	Prognosis and progression	5	Illumina	Yes	28
8-gene expression signature	Recurrence and progression	8	Micromax system	Yes	29
30-gene signature	Prognosis	30	Affymetrix	No	30
Multigene predictor	Prognosis	43	Multiple	Yes	31
34-gene metastasis predictor	High risk of metastasis	34	Affymetrix	Yes	32
23-gene signature	Likelihood of relapse	23	Affymetrix	No	33
Ovarian serous cyst adenocarcinoma					
CLOVAR	Prognosis	100	Affymetrix and Agilent	Yes	34
11-gene signature	Prognosis	11	TaqMan low density array	Yes	35
OCPP	Prognosis	115	Affymetrix	Yes	36
16-gene signature	Prognosis	16	Affymetrix	No	37
Head and Neck					
13-gene signature	Prognosis	13	NA	Yes	38
5-gene methylation signature	Prognosis	5	Agilent	Yes	39
Hypoxia metagene signature	Prognosis	99	Affymetrix	Yes	40
Acute myeloid leukaemia					
24-gene signature	Prognosis	24	NA	Yes	39
86-probe-set gene-expression signature	Prognosis	66	Affymetrix	Yes	41
35-gene signature	Prognosis	35	Affymetrix	Yes	42
133-gene clinical-outcome predictor	Prognosis	133	Stanford Functional Genomics Facility	Yes	43
Skin cancer					
9-gene signature	Prognosis and metastasis	9	qRT-PCR	Yes	44
70-gene signature	Prognosis	70	Research Genetics	Yes	45
254-gene signature	Prognosis	254	Agilent	Yes	46
21-gene signature	Prognosis	21	MWG Biotech	Yes	47
46-gene expression signature	Prognosis	46	NA	Yes	48
Lung cancer					
Yin Yang signature	Prognosis	63	NA	Yes	49
7-gene signature	Prognosis and diagnosis	7	NA	Yes	50
12-gene signature	Prognosis and chemo response	12	Affymetrix	Yes	51
193-gene gene expression signature	Prognosis	193	Affymetrix and Agilent	Yes	52
13-gene signature	Prognosis	13	Affymetrix	Yes	53
21-gene signature	Prognosis	21	Affymetrix	Yes	54
15-gene signature	Prognostic	15	Affymetrix	Yes	55
5-gene signature	Prognosis	5	qRT-PCR	Yes	56
Clear cell carcinoma					
4-microRNA signature	Metastasis and prognosis	4 miRNA	Agilent	Yes	57
5-microRNA signature	Prognosis	5 miRNA	miRXplorer microarray	No	58
34-gene signature	Recurrence	34	NA	Yes	59
40-gene signature	Prognosis and metastasis	40	NA	No	60
microRNA expression signatures	Prognosis	11 miRNA	Affymetrix	Yes	61
Prostate cancer					
7-gene signature plus Gleason score	Prognosis	7	Affymetrix	Yes	62
32-gene prognosticator	Prognosis	32	Illumina	Yes	63
9-gene signature	Prognosis	9	Affymetrix	Yes	64
3-gene prognostic methylation signature	Prognosis	3	NA	Yes	65
11-gene signature	Prognosis	11	NA	Yes	66
Breast cancer					
MammaPrint	Prognosis	70	Agilent	Yes	13
Oncotype DX	Recurrence	21	qRT-PCR	Yes	67
Rotterdam signature	Prognosis	76	Affymetrix	Yes	68
Genomic grade	Histologic grade and tumour progression	97	Affymetrix	Yes	69
Glioblastoma					
G-CIMP	Prognosis	8	Infinium methylation array	Yes	20
9-gene signature	Prognosis	9	Affymetrix	Yes	21
4-gene signature	Prognosis	4	Affymetrix	Yes	22
miRNA signature	Prognosis	10 miRNAs	Agilent	No	23
14-genes signature	Prognosis	14	Real time Q-PCR	Yes	24
9-gene methylation signature	Prognosis	9	Infinium methylation array	Yes	25

patients which included both lymph node-negative and lymph node-positive breast tumour patients[14]. This 70-gene signature is a strong and independent predictor of distant metastasis-free survival (Figure 1) and is the first signature to be cleared by the Food and Drug Administration (FDA)[15]. MammaPrint is currently marketed by Agendia Inc., Amsterdam, The Netherlands.

Oncotype DX breast cancer assay: Oncotype DX is the commercial name of a 21-gene prognostic signature for breast cancer. This signature predicts the likelihood of recurrence of tumour in an early-stage, estrogen receptor (ER) positive breast cancer. Oncotype DX was developed by Genomic Health, Redwood City, CA, USA, and these 21 genes are related to cell proliferation, hormonal response and chemotherapy response. On clinical trials, it was indeed found to be a significant predictor of chemotherapy response and the high-risk patients predicted by the Oncotype DX score were shown to have a better response for tamoxifen plus chemotherapy.

Two gene (HOXB13/IL17BR) expression ratio: This signature was developed after performing a gene expression profiling using a 22,000-gene oligonucleotide microarray[16]. According to this signature, the expression ratio of HOXB13/IL17BR can predict a disease-free survival in patients with early-stage, ER-positive breast cancer who received adjuvant tamoxifen. The assay is carried out using RT-PCR and is marketed by Quest Diagnostic Inc, USA.

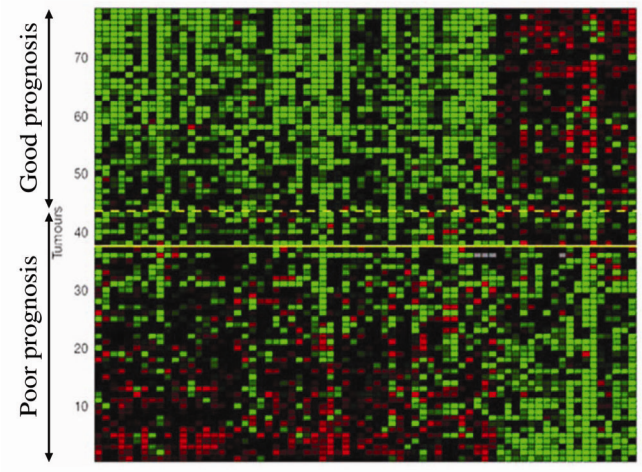

Figure 1. MammaPrint a prognostic gene signature. MammaPrint is an example showing how prognostic signature can be used to identify different outcomes in cancer patients. The heat map represents expression of 70 genes, the score derived from which can divide patients into low and high risk of metastasis. Each row represents a tumour and each column a gene. Solid line, prognostic classifier with optimal accuracy; dashed line, with optimized sensitivity. The colour code indicates that red refers to a higher expression and green indicates lower expression of a given gene. Patients above the dashed line have a good prognosis signature, while those below the dashed line have a poor prognosis signature (adapted from van 't Veer *et al.*[14]).

Colorectal cancer gene signatures

Colorectal cancer is the fourth leading cause of disease-related death in the world[4]. Colorectal cancer can be divided into three groups based on severity of the disease – stages I–III. Similar to breast cancer, many genetic aberrations like microsatellite instability (MSI) and loss of heterozygosity (LOH) of 18q and 17p, etc. have been shown to have prognostic value and can predict the recurrence-free survival in both the malignant tumour stages, but with conflicting results[17]. Though introduction of chemotherapy along with surgery has increased the overall survival of colorectal patients, some of them show signs of complete cure just by surgery and do not need chemotherapy. This led to the development of clinically reliable prognostic markers which can divide the patients into different risk groups and help in taking decision to choose the right type of therapy. Here, we will describe signatures which are currently being used in clinics.

ColoPrint: This is an 18-gene signature which can divide the patients into low- and high-risk groups[18]. ColoPrint was also found to be independent of all other markers and validated in an independent set of patients. The patients with high risk, as identified by this signature, are more prone to recurrence of tumour and are given aggressive therapy. ColoPrint is now marketed by Agendia, USA.

OncoType DX colon cancer assay: This assay, developed by Genomic Health, Redwood City, CA, USA, is composed of 12 genes and predicts the likelihood of recurrence of tumour after surgery, particularly in grade-II tumour patients. Oncotype DX is a multigene real-time PCR based assay, which can be performed using paraffin-embedded tumour specimens. The patients found to be at high risk can be considered for adjuvant chemotherapy to improve their survival.

Promising prognostic signatures for other cancers

Several prognostic signatures for other cancers with great promises have been developed (Table 1). Here, we will discuss the prognostic signatures available for risk assessment in glioblastoma (GBM). GBM is the second most common, next to meningioma, and the most aggressive primary tumour of the central nervous system in adults. Despite all advances in surgery and chemotherapy, the median survival of GBM patients is only 12–15 months[19]. Since all patients do not respond to the existing therapy, patient sub-groups with varying risks need to be identified so that those who belong to low risk may be given the existing therapy, while those who belong to high risk could be considered for more aggressive and multimodal therapy. Towards risk assessment, many prognostic gene signatures that have been developed are described below.

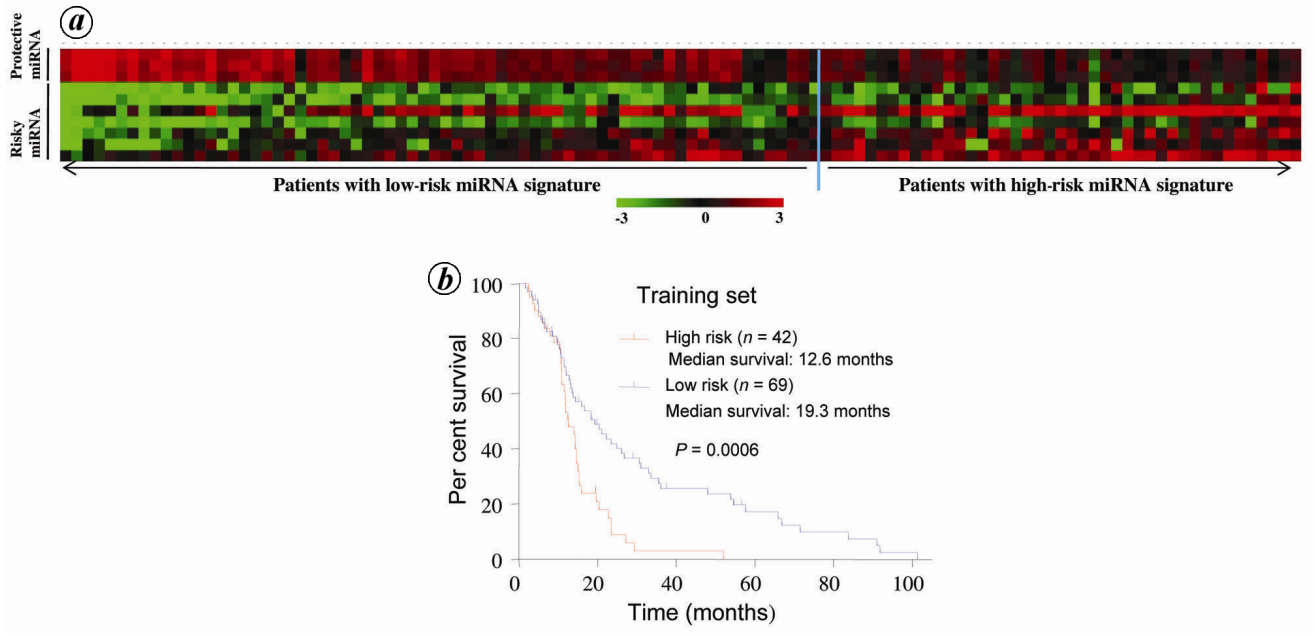

Figure 2. miRNA signature for glioblastoma prognosis. The score derived from expression value of 10 miRNAs is used to divide patients into low and high risk (adapted from Srinivasan *et al.*[23]). ***a***, Heat map of ten miRNA expression profiles of glioblastoma patients; rows represent risky and protective miRNAs and columns represent patients. The blue line represents the miRNA signature cut-off dividing patients into low-risk and high-risk groups. The colour code indicates that red refers to a higher expression and green indicates lower expression of a given miRNA. ***b***, Kaplan–Meier survival estimates overall survival of glioblastoma patients according to the 10 miRNA expression signature. Risk stratification of patients based on risk score divides them into low risk and high risk.

G-CIMP: This refers to glioma-CpG island methylator phenotype, and identifies a sub-group of glioblastoma patients with hyper methylation of a set of genes[20]. These patients are called G-CIMP$^+$ and tend to survive significantly longer than the G-CIMP$^-$ patients. G-CIMP$^+$ tumours have distinct genetic features which include high frequency mutation in iso citrate dehydrogenase 1 (IDH1) and specific copy-number alterations.

9-gene signature: This was developed by Colman *et al.*[21]. They identified 38 genes initially by analysing the microarray data from four different GBM datasets. Subsequent analysis of these genes by quantitative reverse-transcription PCR in another set of GBM patients resulted in the identification nine genes. The 9-gene predictor was found to be an independent predictor of survival and showed positive correlation with markers of glioma stem-like cells, including CD133 and nestin.

4-gene signature: This signature was developed by performing meta-analysis using three different GBM microarray datasets[22]. A risk score calculated based on the expression values of these four genes was found to correlate with survival and also to be an independent predictor of survival in GBM.

10-miRNA signature: miRNAs are small non-coding RNAs, which regulate gene expression post-transcriptionally. We have identified a miRNA signature for GBM prognostication using the dataset derived from The Cancer Genome Atlas (TCGA)[23]. This signature consists of 10 miRNAs, out of which 7 were found to be risky miRNAs and 3 were found to be protective (Figure 2 *a*). The risk score obtained by combining the expression levels of these 10 miRNAs divided GBM patients into low and high risk with significant difference in survival (Figure 2 *b*)[23].

14-gene signature: This signature was also developed by our group using a set of 123 GBM patients who were prospectively recruited, treated with a uniform protocol and followed up[24]. This signature was developed by supervized principal component analysis of the expression of 175 genes determined using quantitative RT-PCR. A weighted gene score derived from the expression of 14 genes was found to be an independent indicator of survival in GBM and was also able to stratify patients into low risk and high risk with significant difference in survival. This study also identified association of activated inflammatory/immune response pathways and mesenchymal subtype in the high-risk group.

9-gene methylation signature: Recently, we have identified a 9-gene DNA methylation signature for prognosis prediction of GBM[25]. This signature was identified by using infinium 27 methylation data of 44 GBM samples, which were then validated in multiple datasets and identified as an independent prognostic signature. The methylation risk

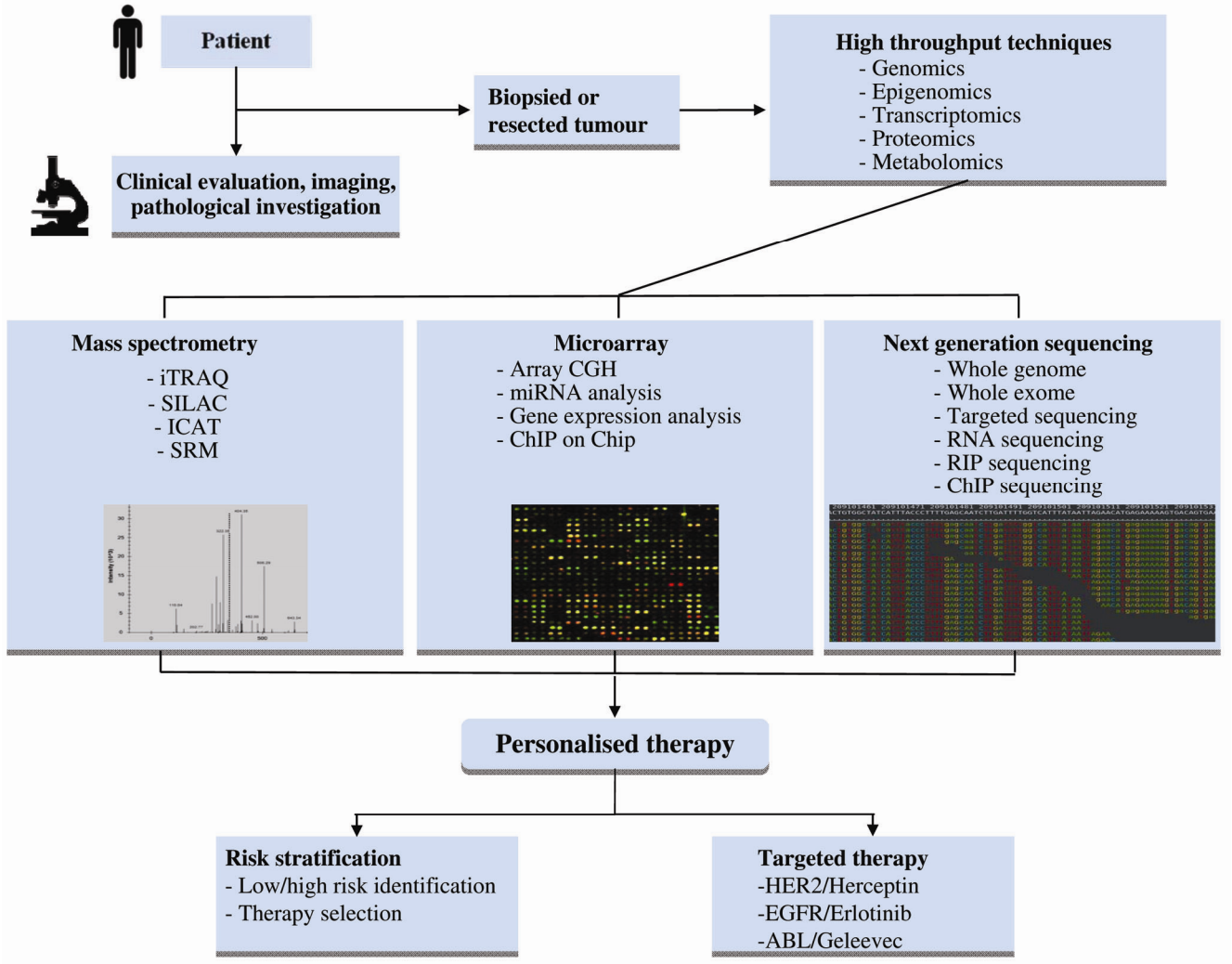

Figure 3. Schematic showing how high throughput analysis can be used in personalized cancer therapy.

score derived from the methylation values of nine genes stratified GBM patients into low and high-risk with significant difference in survival. Using gene interaction network analysis, this study also identified activation of NFkB pathway in high-risk group, thus explaining their poor prognosis.

Next-generation sequencing and personalized medicine

The first draft of the human genome sequence was published 12 years ago. This project, which utilized Sanger sequencing technique, often referred to as the first generation sequencing, took 13 years with 23 laboratories worldwide collaborating at a cost of approximately US$ 3 billion. Now it is possible with the use of next-generation sequencing (NGS), which is actually the second generation sequencing, to sequence a human genome in much shorter time, say, within 10 days and for much less cost, say, US$ 10,000. Since cancer is a disease of the genome, it makes sense to sequence the whole cancer genome to find genetic alterations unique to a tumour so that a tailor-made/patient-specific treatment could be developed. A major advantage of NGS is that one can actually detect all genetic alterations in a tumour at once.

Recent advances in NGS afford new opportunities to uncover specific genetic mutations that drive cancers. This, coupled with the rapid advances in therapeutics, allow targeting these specific mutations in patients, providing precision medicine during the clinical course of disease management. The way it is perceived is that as soon as a patient is diagnosed with cancer, surgically removed tumour tissue or a biopsied tissue material could be subjected to sequencing to quickly determine genetic alterations/mutations that are driving the cancer, based on which an appropriate therapy could be selected.

In practice, NGS is carried out in many different ways. While the high-throughput sequencing of the whole genome (WGS) of a tumour is possible, the whole exome sequencing (WES) provides most of essential information

even though it covers only a part of the genome, which codes for the proteins called exome. Since the exome comprises just over 1% of the human genome, WES is cost-effective with complete information about the protein-coding genes. However, we know now that the part of the genome which is not coding for proteins also appears to play an important role. For example, microRNAs and long non-coding RNAs (lncRNA), single nucleotide variations (SNV) located particularly in the promoter regions have been shown to play important roles. With decreasing cost of NGS, it is anticipated that routine WGS is a real possibility. The advantage with WGS is that it will provide the genetic alterations covering the entire genome in a single exercise.

As against WGS and WES, another approach called 'targeted sequencing' of specific set of genes or genomic regions is also preferred. In addition to affordability due to reduced cost and much shorter time for sequencing, a high coverage could be achieved in targeted sequencing with automatic increase in the quality of the data. Many genetic testing laboratories have started including targeted sequencing of gene panels in their routing laboratory testing. For example, ONCOSeq panel, offered by Rain Dance Technologies, USA, utilizes targeted sequencing approach to investigate 142 selected cancer genes. Foundation One, a targeted sequencing test offered by Foundation Medicine, USA, sequences 236 cancer-related genes that are associated with cancer-related pathways, targeted therapy or prognosis.

There are also certain limitations currently in the use of NGS in medicine. The cost of sequencing is not yet affordable, although it is expected to come down to few a hundred US dollars per genome in the coming years. Further, creating a NGS facility could easily cost up to several hundred thousand US dollars. Sequencing errors which may arise due to repeat sequence region and short read lengths would be a problem. NGS data analysis requires specially training personnel with bioinformatics knowledge and is time-consuming.

Conclusion and future perspectives

While the output from microarray and NGS-based high-throughput techniques is highly promising, it is important to know that they are not meant to replace but only to complement the conventional clinical and pathological studies. Significant technical advancements have been made in the field of cancer diagnostics and therapeutics, but these suffer with serious lacunae. While gene signatures are already in use in breast and colon cancer for risk identification, we stand today with a plethora of molecular signatures with very few of them making it to the clinical trials. This may be due to the dissonance of molecular noise that we obtain from the omics studies. There are many hurdles before the signatures could be ready for routine use in the clinic. One of the most important requirements is the external validation of signatures using multiple institutions with large cohorts. It is also important to understand the biology behind the gene signatures as this might help in developing alternate therapy for high-risk group patients. Some of the other factors which may influence the success in routine use of signatures are: high cost involved, the use of different platforms for generating the signatures and the requirement of skilled personnel to carry out the work.

While there are many successful examples of targeted therapies based on specific genetic alterations, there are many hurdles before NGS can be implemented into routine use for patient care. It remains to be tested to find which of the approaches – targeted sequencing of selected genes or WES or WGS is more suitable and commercially viable. WGS is likely to identify a large number of genetic alterations with unknown functions, which will make the information unusable. Accuracy of mutation detection by NGS is another big challenge as the current method of data analysis is highly error-prone. While deep sequencing can overcome this, there are problems like the presence of stromal cells and increase in time and cost of the analysis. Another key challenge is the lack of physician and patient understanding of NGS-derived data. Hence there is a great need to educate people involved and also develop tools for clinical decision support which will integrate the NGS data to the practice of medicine.

In the era of WGS and other high-throughput omics approach, including genomics, transcriptomics, proteomics and metabolomics, we can easily envisage a future wherein these approaches will be used in combination with current therapies and can help overcome the molecular heterogeneity and resistance observed in some classes of tumours.

To conclude, we are in an extremely exciting era with an ability to characterize the entire genome of both tumour and patient. There is a huge promise that these technologies will provide unique targets based on which specific therapies more suitable for a given patient could be developed. However, there are many challenges that need to be overcome before the tailor-made cancer therapies are possible.

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