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Emerging Role of Next Generation Sequencing (NGS) in Treatment of Solid Tumors

Maher Salamoon*

Department of Medical oncology, Al Bairouni University Cancer Center, Damascus, Syria

***Corresponding Author:** Maher Salamoon, Department of Medical oncology, Al Bairouni University Cancer Center, Damascus, Syria. Email: Maher.salamoon@gmail.com*

THE ERA OF NGS

Immunohistochemistry (IHC) played a major role in cancer treatment decision during the last decade through introducing a new classification for cancers depending on protein expression on cancer tissue. Molecular testing became as well a reliable method in detecting mutations through polymerase chain reaction (PCR) and gene amplification through Fluorescence in situ Hybridization (FISH) or chromogenic in situ hybridization (CISH). Sanger sequencing method paved the road to NGS to take the lead as the method of choice in detecting multigene/variant assessment. NGS can detect DNA mutations on circulating tumor DNA and circulating tumor RNA as well. It can performed on paraffin embedded tissues, blood and bone marrow as well. NGS needs to run in special well equipped laboratories specialized in molecular biology with a very strict quality control beginning from tissue preparation and not terminating in bioinformatics and final interpretation.

SITE-AGNOSTIC BIOMARKERS

Microsatellite Instability (MSI)

During DNA replication, some errors occur (i.e, single base mismatches, insertions and deletion) which are corrected by the mismatch repair mechanism (MMR). Polymerase is able to correct errors by excising them and inserting the suitable matches instead (1). Cells accumulate errors during DNA replication when MMR loses its function leading to a novel microsatellite formation. Microsatellites are repeated sequences of DNA which can be made of repeating units of one to six base-pairs. MSI structure consists of repeated nucleotides most often GT/CA repeats (2). In some tumors, MSI can be caused by a germ line mutation in one of the MMR genes (MLH1, MSH2, MSH6 or PMS2) resulting in Lynche syndrome. However, most of MSI cases are sporadic (80%) due to hypermethylation of MLH1 promoter (3,4). MSI-high (MSI-H) tumors are more sensitive to treatment than those with MSI stable tumors, and most of these MSI-H tumors are sensitive to PD-1/PDL-1 inhibitors (5,6). Recently, Pembrolizumab which is a PD-1 inhibitor, was approved by the FDA for the treatment of MSI-H cancers. Now it is approved for the treatment of metastatic colorectal carcinoma after progression on 5-Fluorouracil, Oxaliplatin and Irrinotecan and for the treatment of Non-Small Cell Lung Cancer (NSCLC) in the first line (7,8). Nivolumab which is another PD-1 inhibitor, was granted an accelerated approval by the FDA to treat MSI-H and dMMR colorectal cancers (CRC) in both pediatric and adults in 2017.

In 2018, FDA granted the combination of Nivolumab and Ipilimumab (CTLA-4 inhibitor) an approval in the same former patient group (9, 10).

Neutrophic Receptor Tyrosin Kinase (NRTK)

NRTK1/NRTK2/NRTK3 family are fusion oncogene found in rare pediatric and adult cancers including NSCLC, CRC and head and neck cancers (11). NRTK1/NRTK2/NRTK3 encodes for neurotrophin receptor kinase A [TRKA], TRKB, TRKC respectively which are found to be expressed in aggressive gliomas. In November 2018, the FDA has granted an accelerated approval for the selective oral TRK inhibitor Larotrectinib to be used in solid cancers harboring NRTK fusion without a known acquired resistance mutation in both pediatric and adult population (12).

Germline Mutations

Germline mutations or alterations are present at birth and in order to discover them, DNA should be extracted from the white blood cells, buccal mucosa and skin fibroblasts in culture. Some somatic mutation may reflect germline changes (i.e, TP53 mutations are found in more than

60% of lunge cancer and at the same time they are found to be mutated in LI-Fraumini syndrome (13).

dMMR is found in 12% of colon cancers and 25% of which are found to be inherited (14). BRCA1/BRCA2 are found to be mutated in both breast and ovarian cancer, furthermore, they can be found mutated in other cancers such as pancreatic adenocarcinoma. In a series study of 100 patients with pancreatic carcinoma, 7 patients had mutations in BRCA2 and 4 of which in the germline which reflects the importance of performing the genetic testing for the family members (15).

Specific Biomarkers by Tumor Site

Colorectal Cancers

Genomic testing is highly recommended in CRC however the timing is still a matter of debate depending on the stage of disease and the test purpose as well. It is reasonable to perform the test at the initial diagnosis of stages I, II and III especially MSI because those with MSI-H have better prognosis and they should go for adjuvant chemotherapy (16). There is a growing evidence that 5-Fluorouracil (5-FU) and Capecitabine may worsen patient outcome when given as a single agent in early stage MSI-H disease (17). Guidelines highly recommend the genetic testing for all stages of CRC to rule out Lynch syndrome and to guide choices of therapy as well. Aspirin was found to reduce premalignant polyp formation in both patients and their relatives with MSI-H tumors (18). Aspirin improved outcome in patients with tumors harboring PIK3CA mutations (19). Several genes may predict outcome of locally advanced CRC such as : BRAF, HER-2, KRAS, NRAS, NTRK, POLE, PIK3CA, PTEN and RSP03 and it is recommended when the disease becomes refractory. Patients with NTRK fusion are good candidates for larotrectinib (12). In the other hand, patients with mutated RAS are insensitive to treatment with anti-EGFR monoclonal antibodies such as cetuximab or panitumumab (20). BRAF V600E is the most widely known mutation in metastatic CRC and it is considered a strong predictor of overall survival (OS) and patients with this mutation have a poor prognosis and short OS (21). BRAF mutated patients prone to be females with old age, high grade right sided tumors with MSI-H. those patients have a high rate of lymph nodes involvement as well (22). Gene profile of colorectal cancer is illustrated in table (1) while the most frequent genes are shown in table (8).

Gastric and Esophageal Cancers

Those kind of tumors express Her-2 which can be confirmed by Fluoroscence in situ hybridization (FISH) and Trastuzumab is indicated in these cases. However, patients with negative Her-2 status and dMMR tend to highly express PD-L1 making them good candidates for anti-PD-L1 therapy, therefore, FDA has granted pembrolizumab an approval in patients with gastric cancers expressing PD-L1 (23). The most important genes are illustrated in table (3)

Pancreatic Cancers

9% of pancreatic adenocarcinomas harbor a germline or a somatic mutation in BRCA1 or BRCA2, therefore, BRCA testing by NGS is becoming a standard practice in patients who are still responsive to chemotherapy. Germline mutation n BRCA1 or BRCA2 can guide therapy using poly adenosine di-phosphate-ribose polymerase (PARP) or DNA damage repair (DDR) enzymes inhibitors. Olaparib was approved in October 2018 to treat mutated BRCA pancreatic adenocarcinoma that has not progressed on first line platinium based chemotherapy (24). The most importany genes are illustrated in table (3).

Bladder Cancers

Robertson et al , in a genomic study on 412 patients with muscular layer invasive bladder cancer found mutations in DNA repair genes prevalent as follows: ERCC2 (10%), ATM (40%) and RAD51B (2%) (25). ERCC2 mutations were mostly missense and were associated with improved response to platinum based chemotherapy. In preclinical models, loss of ERCC2 was enough to trigger tumor sensitivity to Cisplatin, that is why, ERCC2 can be used as a predictive biomarker of response to chemotherapy (27).

Prostate Cancer

In patients with metastatic castration-resistant prostate cancer (mCRPC), Antonarakis et al reported better clinical outcomes after 1st line treatment with abiraterone and enzalutamide in patients with germline mutation in BRCA1/BRCA2 and ATM (28). In a separate study, responders to PARP inhibitors were found to have a mutation in BRCA2 (29). In 2018, De Bonno et al, showed that pembrolizumab showed a good response rate and an acceptable disease control in Docetaxel-refractory mCRPC irrespective of PD-L1 status (30). The response rate was better in those with

somatic mutations in BRCA1/BRCA2 and ATM as well making them good predictive biomarkers to check-point inhibitors. Of note, National Comprehensive Cancer Network (NCCN) recommend BRCA1/BRCA2 testing, however, ATM is suggested but not recommended yet (31) especially with a known family history of prostate, colon, pancreas and Lynch syndrome. Those persons are at increased risk for developing prostate cancer and BRCA1/BRCA2 testing is highly suggested. The most important genes encountred in prostate cancer are illustrated in table (9).

Endometrial Cancers

Lynch syndrome may be associated with 2-5% of endometrial cancers that may be caused by a germline mutation in MLH1, MSH2, MSH6 and PMS2. In the absence of germline mutation, MSI-H tumors can be caused by a hypermethylation in MLH1 gene. MSI-H recurrent endometrial carcinomas caused either by a germline mutation or a hypermethylation are treated with pembrolizumab based on site-agnostic FDA approval granted for the molecule in 2017 (6). POLE-aberrant cancers have a theoretical good prognosis, however, discovering hotspot mutation in several genes including BRAF, KRAS, PIK3CA and PTEN may predict the prognosis and biological behavior rather than treatment guidance. ESR1 (ER) expression has an important value in stage III and IV as well as in recurrent disease predicting response to endocrine therapy.

Ovarian Cancers

One of the most frequent gene mutated in ovarian cancers is BRCA-related genes which can predict a serous epithelial cancers responding to platinum compounds and PARP inhibitors. The spectrum of studied genes is very vast including BRCA1, BRCA2, BARD1, BRIP1, PALB2, MLH1, MSH2, MSH6, PMS2 and STK11. Patients with mutations in the former genes, have a good prognosis and sensitive disease to treatment with platinum agents. BRCA1/BRCA2/ATM mutations are studied at initial diagnosis in those with a family history and in recurrent disease because those two genes along with MSI / DNA MMR can help predicting response to PARP inhibitors and to chemotherapy as well. Other genes such as BRiP1, CHEK1, PALB2 and RAD51C/ RAD 51D are studied at diagnosis in patients with family history and/or recurrent disease (32). Based on the improvement in progression free survival rate (PFS) concluded from the SOLO-1 trial, mutations in BRCA genes recommend maintenance treatment with PARP inhibitors after the upfront chemotherapy with platinum and paclitaxel (33). As a secondary conclusion from SOLO-1 trial, it was observed that a reduction of 70% in ovarian cancer progression was observed in women with germline or somatic mutation BRCA who received the Olaparib maintenance. However, women without BRCA mutation showed better course by addition of Bevacizumab to the upfront carboplatin and paclitaxel (34). For recurrent ovarian cancers with proven BRCA1/BRCA2 mutation, Olaparib, Niraparib and rucaparib were approved as a switch maintenance therapy for those who responded to platinum based chemotherapy in the second and third line setting (35). Based on agnostic-site label, pembrolizumab was approved in MSI-H tumors however, this agent has a modest activity when used alone, therefore, a combination between PD-1 inhibitors and PARP inhibitor in several trial. The largest on is ATHENA trial which is still recruiting patients evaluating the combo Rucaparib and Nivolumab as maintenance therapy in ovarian cancer patients who responded to front line platinum based chemo. Table (4) illustrate gene profile of ovarian cancer with the most frequent genes expressed in table (12).

Cervical Cancers

Recurrent cervical cancers have a very bad prognosis with a poor outcome. Gene profile is poorly studied with few genetic biomarkers that may guide treatment. The anti-angiogenic Bevacizumab was approved in recurrent setting in combination with platinum, paclitaxel and topotecan, however, survival rate is still disappointing. In patients with recurrent and metastatic disease previously treated with a combination chemotherapy, the FDA approved pembrolizumab in such cases expressing PDL-1 based on a KEYNOTE 158 study; clinicaltrials. gov , NCT 02628067 which demonstrated an objective response of 14%. Nivolumab as a single agent has shown a response rate of 26% (clinicaltrials.gov identifier NCT02488759).

Breast Cancer

In newly diagnosed and recurrent breast cancers, the most important players in decision making are: Estrogen receptors (ER), Progesterone receptors (PR) and Epidermal growth Factor Receptor status (Her-2). The newly investigated biomarkers are Androgen Receptors (AR), ESR1 and PDL-1. In some subset of triple

negative breast cancer (TNBC), androgen receptors are highly expressed which led to a designation of androgen targeted clinical trials which in turn showed a promising results in metastatic TNBC (36). De novo mutations in ESR1 take place in the ligand-binding domain of ER leading to resistance to aromatase inhibitors. Those mutations are found during and after treatment of ER positive breast cancers, therefore, ER down regulators will be one of the best choices in this setting. In early breast cancers, several genomic assays are employed to guide treatment option such as Oncotype DX, Mammaprint and Prosigna. Mammaprint and Prosigna are used as prognostic parameters for relapse in patients with negative nodes, who have between 1-3 positive nodes or are ER positive but Her-2 negative (37). BRCA1/BRCA2 mutations are predictor of response to PARP inhibitors. Several genes will be important very soon in both treatment guidance and response prediction to new agents, and those genes include : TP53, ATM, NBN, RAD51c, RAD51D, NF1, BRIP1, SKT11, MUC16, PTEN, CDH1, CHEK2, PALB2CDK4/6, EPCAM deletion, MLH1,MLH2, MSH6 and PMS2. Table (10) illustrate the most important genes expressed in breast cancer.

Central Nervous System Cancers

In central nervous system cancers, genetic alterations are diagnostic rather than prognostic or predictive. The World Health Organization (WHO) classifies nervous system tumors depending on histologic subtypes, however, more than one subtype may co-exist in the same tumor leading to the diagnosis of a hybrid oligoastrocytoma(38). However, histologic studies along with gene profiling can guide pathologists to differentiate between oligodendroglioma and astrocytoma. With consideration of IDH mutation, 1p19q codeletion, ATRX loss and TP53 mutation, the WHO modified histologic classification by combining the molecular genetic biomarker with histopathology (39). The methyl-guanine methyl transferase gene MGMT is routinely tested because it is an important prognostic factor and predictor of response to alkylating agents (40). A recent NGS based genomic study on 43 patients using a panel of 315 genes, a median of 4.5 gene alterations per patient were detected and the most frequent was in EGFR gene (40%) and the genotype directed treatment in 13 patients (30%). However, treatment with everolimus, erlotinib, afatinib, palbociclib and trametinib showed no response (41). High grade gliomas are driven by the fusion Brevican (BCAN) and NTRK1and it was found that such tumors are sensitive to entrectinib (42). BRAF V600E was detected in 17% of pediatric low grade gliomas patients. Those patients have a very poor outcome on chemotherapy (43). For this reasons, several new drugs were evaluated in pediatric low grade gliomas (refractory or in relapse) and the most exciting results came from the phase 1 / 2 trial of dabrafenib that demonstrated 38% of response rate and another 44% patients with stable disease (44). Table (5) demonstrate gene profile in glioblastoma while the most frequent genes are illustrated in table (11)

Sarcomas

sarcomas have different subtypes with histologic and biologic differences between adult and pediatric disease. The driver mutations differ from one type to another (i.e, EWSR1-FLI1 in Ewing sarcoma, PAX3/PAX7-FOX01 in alveolar rhabdomyosarcoma, SYT-SSX2 in synovial sarcoma, TLS-FUS/CHOP in myxoid liposarcoma. Those former fusion can be detected by FISH and they could be a possible targets for new molecules.

Head and Neck Cancers

PD-1 test is highly recommended in head and neck squamous cell carcinoma (HNSCC) which is highly expressed in these tumors. Based on this finding, both nivolumab and pembrolizumab were approved for the treatment of refractory or relapsed HNSCC after treatment with platinum based chemotherapy (45,46). EGFR is overexpressed in 90-100% of patients with HNSCC and accordingly cetuximab was approved in this setting irrespective of EGFR mutation testing (47). Several genes play a major role in resistance to anti-EGFR agents including insulin like growth factor IGF, c-met and PI3K (48). also is of value to differentiate between human papillomavirus (HPV) positive and negative tumors since patients with HPV positive cancers are more responsive to chemotherapy(49).

Melanomas

About 50% of melanomas originating from cutaneous sites harbor BRAF V600E mutation. BRAF genotype was approved by the FDA as the only predictive biomarker in advanced melanoma (50). BRAF activation leads to activation of MAP kinase pathway and increasing proliferation an cell survival. Several drugs were designated to target cells with BRAF

V600E mutation including vemurafinib, darbafinib and encorafenib, however, it is of value to know that BRAF inhibition may cause a huge MAPK activation in cells with BRAF wild type through BRAF dimerization and consequent cell proliferation (51). For patients with BRAF V600 mutation, the FDA approved the combination of selective BRAF inhibitors and MEK1/MEK2 inhibitors. The combination of dabtafinib and trametinb showed to improve relapse free survival in patients with resectable stage III disease with BRAF V600E/V600K mutation (52,53).

Similarly, the same former combination demonstrated an objective response of 68% in patients with unresectable advanced melanoma harboring BRAF V600E/K mutations. Another gene complicated in melanoma carcinogenesis is kit where mutations take place across several exon. Kit mutation is observed in 20% of advanced melanoma patients with approximately 29% response rate on imatinib (54) with maximal response seen in melanomas harboring mutations in Kit exon 11 and 13. Another important oncogen is NRAS which is found mutated in about 20% of patients with advanced melanoma, however, it is hard to target this oncogene, therefore, efforts were made to work understream by targeting MEK1/MEK2 through binimetinib that demonstrated good clinical response but it has not been approved by the FDA yet (55). Interestingly, PD-1/PDL-1 studies became more important in melanoma and nivolumab demonstrated a response rate of 58% in patients whose their tumors stained > 5% for PD-1 versus only 43% in those with a staining less than 5%. In the checkmate 067 study, the combo nivolumab and ipilimumab demonstrated an improvement of progression free survival versus nivolumab alone in BRAF v600E with PD-1 staining less than 1% (hazard ratio 0.62 vs. 0.68 for nivolumab alone) (56). Of note, it should take into consideration that there is a direct proportion between the high mutation burden and response to PD-1/PDL-1 inhibitors and the more the mutational score per kilobases the more the response.

Lung Cancers

Concentration has been made on treatment of adenocarcinoma of the lung because of the several genetic alterations encountered during the course of disease. One of the most important gene implicated in adenocarcinoma carcinogenesis is the epidermal growth factor receptor (EGFR) the former gene is found in 15-20% in Caucasian patients versus 30-40% in Asians (57). Mutations in exons 19 and 21 is treated by the first and second generation EGFR tyrosine kinase inhibitors such as gefitinib, erlotinib and afatinib (58). However, within the first 8-12 months of treatment relapse occur and the most common acquired mutations leading to resistance are exon 19 deletion, L858R mutation and T790M which is found in about 50-60% of resistant cases (59). For this issue, the third generation EGFR tyrosine kinas inhibitor osimertinib was approved by the FDA for the first line treatment of adenocarcinoma of the lung with EGFR ex 19 deletion or ex 21 L858R. Full approval of osimertinib was granted by the FDA to treat adenocarcinomas progressed on first generation EGFR inhibitors and demonstrated T790M mutation (60). Some other genes such as ALK and ROS are attracting more attention because of their fundamental role in adenocarcinoma development and new agents are approved such as crizotinib for ALK and ROS positive disease. Efforts also have been made to overcome resistance developed by C797S mutation (61).

Conclusion

NGS is considered to be the bridge between molecular testing and clinical practice. However, genetic testing has not become a routine practice except limited examples recommended by the NCCN such as the pan RAS testing in colorectal cancers, Her-2 in breast cancer and the EGFR mutation profile in adenocarcinoma of the lung. Huge data was gathered from trials including patients having the former mutations in order to conclude results guiding us in our daily practice. Genetic data is growing day after day, however, we should concentrate on the most frequent mutations per cancer type especially targetable ones. NGS will play a major role in guiding the clinical applications of newly designed drug taking into account the cost effectiveness and the benefit we can get from the new related medications.

Table1. *Genetic profile in colorectal cancer*

Biomarker	Technology	Evidence
MMR	IHC	Wide acceptance
MLH1, MSH2, MSH6, PMS2	NGS	Wide acceptance
MSI	PCR, NGS	Wide acceptance

Table2. *Gene profile of NSCLC*

Biomarker	Technology	Evidence
ALK	Fish, rt-PCR,NGS	high
EGFR T790M	NGS	high
EGFR ex 18-21	NGS	high
EGFR ex 19 del	NGS	high
EGFR ex 20 ins.	NGS	high
ROS1	NGS,FISH,PCR	low
PDL-1	NGS	low
KRAS	NGS	low
BRAF	NGS, PCR	low
Met/Ret	NGS,FISH	low
HER-2	NGS, FISH	low

Table3. *Gene profile of gastric and pancreatic cancer*

Biomarker	Technology	evidence
Her-2	FISH	Low
PDL-1	IHC, FISH	Low
BRCA pancreas	NGS	high

Table 4: *gene profile of ovarian cancer*

Biomarker	Technology	Evidence
BRCA1/BRCA2	NGS	Low, wide acceptance
ATM	NGS	Low, wide acc
BRiP1	NGS	Low, wide acc
CHEK2	NGS	Low, wide acce
PALB2	NGS	Low, wide acc
RAD51C, RAD51D	NGS	Low, wide acc

Table5. *Gene profile of CNS cancer*

Biomarker	Technology	Evidence
1p19q codeletion	FISH, PCR	Low
IDH1, IDH2	NGS, IHC	Low
MGMT	PCR	low

Table5. *Gene profile in sarcoma*

Biomarker	Technology	Evidence
MDM2, CDK4	NGS	Wide acceptance
IDH1, IDH2	NGS	Wide acceptance

Table6. *Gene profile in melanoma*

Biomarker	Technology	Evidence
MDM2, CDK4	NGS	Wide acceptance
IDH1, IDH2	NGS	Wide acceptance
	Head & neck	
PDL-1	IHC	low

Table7. *Percentage of mutant genes in NSCLC*

	NSCLC	
Biomarker	**Technology**	**percent**
PDL-1	NGS	45
PTEN	IHC	62
KRAS	NGS	28
EGFR L858R	NGS	3.7
EGFR ex 19 del	NGS	5.6
EGFR T790M	NGS	1.3
EGFR tertiary	NGS	0.1
ALK	NGS	0.2
ROS1	NGS	0.3

Table8. *Percentage of mutant genes in colorectal cancer*

	Colon	
Biomarker	**Technology**	**percent**
KRAS	NGS	50.9
NRAS	NGS	4.3
BRAF	NGS	8.3
MLH1	IHC	95
PMS2	IHC	93
MLH6	IHC	97
PDL1	IHC	3.1
HER-2	CISH	2.9

Table9. *Percentage of mutant genes in prostate cancer*

	PROSTATE	
BIOMARKER	**TECHNOLOGY**	**PERCENT**
AR	IHC	92
PDL-1	IHC	3.2
TMPS2:ERG	FUSION	28
ATM	NGS	5.2
BRCA2	NGS	7
MSI	NGS	4

Table10. *Percentage of genes expressed in breast cancer*

	BREAST	
AR	IHC	51
ER	IHC	59
PR	IHC	40
HER-2	CISH	11.9
PT53	NGS	55.5
BRCA1	NGS	3
BRCA2	NGS	4.5
PDL-1	IHC	7
PTEN	NGS	6.6

Table11. *Percentage of genes expressed in glioblastoma*

	GLIOBLASTOMA	
IDH1	NGS	18.9
IDH2	NGS	0.4
MGMT	METHYLATION	46.7
1P199Q	FISH	6.7
PDL-1	IHC	15
EGFR	CNV	30
MSI	NGS	0.4
BRAF	NGS	4

Table12. *Percentage of genes expressed in ovarian cancer*

	OVARY	
BRCA1	NGS	8.1
BRCA2	NGS	6.2
PIK3CA	NGS	8.4
KRAS	NGS	7.3
ER	IHC	48
PR	IHC	28
PDL-1	IHC	8.6

Table13. *Percentage of genes expressed in melanoma*

	MELANOMA	
MSI	NGS	0
TML	NGS	37

BRAF	NGS	40
KIT	NGS	2
NF1	NGS	24
NRAS	NGS	25
CDKN2A	NGS	14
PDL-1	IHC	33

Table14. *Percentage of genes expressed in uveal melanoma*

	UVEAL MELANOMA	
BAP1	NGS	49
GNA11	NGS	43
GNAQ	NGS	48
SF3B1	NGS	11
PDL-1	IHC	15
TML	NGS	3.1
MSI	NGS	0

Table15. *Percentage of genes expressed in sarcoma*

	SARCOMA	
RB1	NGS	6.1
NF1	NGS	4.9
EWSR1	NGS	10.7
PDL-1	IHC	20.9
RAF-1	NGS	3.6
NTRK-1	NGS	0.2
ALK	NGS	0.6

Of note, the national cancer institute is running the match trial which is recruiting patients with a known targetable gene alterations using new molecules ranging from new generation of tyrosin kinase inhibitors, immune check point inhibitors to targeted monoclonal antibody. As a final conclusion, NGS is opening the door for a new kind of research by a new type of researchers because using and manipulating this technology necessitate certain level of education.

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