ONCONEXT LIQUID™ LUNG

Monitor

LIQUID BIOPSY FOR MONITORING OF CANCER: ANALYSIS OF HOTSPOT MUTATIONS VIA PLASMA DERIVED CIRCULATING TUMOR DNA

Cancer and somatic mutations

The majority of cancers arise after a series of somatic gene mutations that accumulate during an individual's lifetime[1,2]. Somatic mutations occur spontaneously and can originate in any type of cell. These alterations may result from errors during DNA replication or from contact with detrimental environmental factors due to accidental or occupational exposures, or life style choices. Unlike inheritable pathogenic variants found in the germline, acquired somatic mutations are not passed on from one generation to the next.

Thousands of somatic mutations that can drive cancer progression, metastasis, treatment response and resistance have been catalogued by researchers and clinical laboratories. Identifying and understanding the somatic alterations in an individual's tumor can be crucial in cancer diagnosis and in planning personalized cancer treatment, monitoring response to therapy, and identifying cancer recurrence. Moreover, as a tumor progresses, it continues to acquire additional alterations that can affect the response to therapeutic agents such as chemotherapy or targeted therapies.

Established methods including fine needle aspiration, tissue biopsy, microscopic examination, in situ DNA or RNA hybridization, and immunohistochemical stains are routinely used to identify somatic changes in various cancer types. However, a tissue biopsy is an invasive procedure that can be difficult to obtain, and frequently involves co-morbidities for the patient. Moreover, it is costly to perform sequential tissue biopsies to assess residual tumor burden and changes in genetic composition during and after cancer treatment[3,4]. Also, assessment by a tumor biopsy is only a viable option when the primary sites of the tumor or metastases are known and surgically accessible.

Novel methods of somatic mutation analysis, known collectively as a **liquid biopsy**, measure cell-free DNA (cfDNA), more specifically **circulating tumor DNA** (**ctDNA**), or **circulating tumor cells** (**CTC**) from a blood sample. **Plasma** obtained from the blood sample, instead of tumor tissue obtained via a biopsy or fine needle aspiration, now allows non-invasive, highly sensitive and specific detection of somatic mutations in various cancers.[36-39]

Circulating Tumor DNA (ctDNA)

All cells, including tumor cells and non-malignant cells, shed DNA, called **cell-free DNA** (**cfDNA**), into the circulatory system (Diaz and Bardelli 2014). Cancer and other conditions, such as renal failure and myocardial infarction, often result in higher levels of cfDNA than in healthy patients (Diaz and Bardelli 2014). **Circulating tumor DNA** (**ctDNA**) is cfDNA that is shed from tumor cells into the circulatory system[4]. The mechanisms whereby tumor cells release DNA into the blood are not well understood; DNA may enter the bloodstream via secretion from viable tumor cells as either free DNA or in cell-derived vesicles known as exosomes, via secretion from phagocytes post-engulfment of tumor cells, or as a result of tumor cell death through necrosis and apoptosis (Aarthy et al. 2015; Chaudhuri et al. 2015; Diaz and Bardelli 2014; Ignatiadis, Lee, and Jeffrey 2015; Polivka, Pesta, and Janku 2015). Once in the bloodstream, the DNA persists only for a short time ($t_{1/2}$ of ~2 hours) (Diaz and Bardelli 2014; Diehl et al. 2008). Most cfDNA and ctDNA are between 180-200 base pairs (bp) in length (Diaz and Bardelli 2014; Diehl et al. 2005; Diehl et al. 2008; Fan et al. 2008; Jahr et al. 2001; Mouliere et al. 2011). ctDNA can be distinguished from other cfDNA by the presence of somatic mutations, but in the case of solid malignancies ctDNA makes up only a **small fraction** (often only **<1%**)(Diehl et al. 2005; Diehl et al. 2008; Holdhoff et al. 2009); in hematological malignancies (e.g., leukemia), on the other hand, the blood

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contains much higher percentages of cfDNA derived from cancer cells. The contributing fraction of ctDNA to the total cfDNA increases with increasing tumor burden (Diaz and Bardelli 2014; Diehl et al. 2008; Newman et al. 2014) and, as such, the amount recovered may vary greatly among patients.

What is a liquid biopsy?

The term "***liquid biopsy***" describes non-invasive, highly sensitive and cost effective methods of isolating and detecting these cfDNA fragments, including circulating tumor DNA (ctDNA), from the plasma of patients diagnosed with cancer or from individuals who may have cancer. Liquid biopsies are thought to capture the entire tumor genome[5,6]. When liquid biopsy techniques are combined with deep sequencing technologies, a new set of tools is created that identify somatic genomic alterations in tumors. These data can subsequently be used for personalized treatment and monitoring[7-9].

Thus, by analyzing cell-free DNA isolated from a patient's blood, we can identify clinically relevant genomic alterations in ctDNA and match these alterations to targeted therapies and clinical trials.

Liquid biopsies offer a potential alternative to surgical tumor biopsy and histological assessment, eliminating many of the difficulties and concerns associated with traditional tests (**Table 1**) as well as a means of augmenting imaging studies and other diagnostic methods.

Given these advantages, as more studies are reported demonstrating the correlation between mutations in tumor tissue and ctDNA, ctDNA may have an increasing utility in the clinical setting for the investigation of solid tumors as a diagnostic and prognostic tool (Aarthy et al. 2015; Chaudhuri et al. 2015; Diaz and Bardelli 2014; Ignatiadis, Lee, and Jeffrey 2015; Jovelet et al. 2016; Polivka, Pesta, and Janku 2015). Because tumors are temporally and spatially heterogeneous (Gerlinger et al. 2012; Hiley et al. 2014; Ichihara and Lovly 2015; Nik-Zainal et al. 2012; Piotrowska et al. 2015; Wang et al. 2014), a tissue biopsy may only give a "snapshot" of one portion of one tumor at one time. Considering that blood collection is easier than a tissue biopsy, and the fact that ctDNA likely derives from all tumor sites, liquid biopsy has the potential to more accurately monitor a patient's disease burden and progression in real time by allowing detection of DNA characterizing intra-tumor and inter-tumor heterogeneity (Aarthy et al. 2015; Chaudhuri et al. 2015; Diaz and Bardelli 2014; Ichihara and Lovly 2015; Ignatiadis, Lee, and Jeffrey 2015; Piotrowska et al. 2015; Polivka, Pesta, and Janku 2015). Further, ctDNA has been detected in a number of other bodily fluids, such as urine, stool, cerebrospinal fluid, and saliva; so ctDNA testing may involve tumor monitoring utilizing these sample sources as well (Patel and Tsui 2015).

Table 1: Advantages of liquid biopsy over tumor biopsy

Tumor Biopsy	Liquid Biopsy
Invasive and expensive	Non-invasive and less expensive
Specific to localized tumor site	Less dependent on original tumor site since tumor from both primary and metastatic sites release DNA into the bloodstream
Assessment of tumor heterogeneity limited to section of biopsy analyzed	Can capture tumor heterogeneity
A limited amount of tissue may be obtained for immunohistochemical and genomic analysis	A few copies of mutant ctDNA are sufficient for analysis
Difficult to biopsy some organs	Easy to collect sample from blood
Not viable if primary tumor has been resected or if the tumor cannot be easily visualized via imaging studies	Allows for serial evaluation in absence of detectable primary tumor or metastases
Serial biopsies are difficult to tolerate	Patient can tolerate serial blood draws for evaluation; may lead to greater compliance
	New tool that can be applied for evaluation of response to therapy and for detection of residual disease
	May allow for evaluation of development of resistance
	May aid in early detection of cancer

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The Clinical Utility of ctDNA in Monitoring Cancer and Directing Therapy

Research has shown that somatic mutations in a defined set of genes are often the underlying drivers of the development of cancer across different tumor types (**Table 2**)[1]. These genes include, but are not limited to, *BRAF*, the *RAS* gene family, *EGFR*, *PIK3CA*, *FOXL2*, and *TP53*. Somatic mutations in *BRAF* gene are commonly associated with malignant melanoma, non- Hodgkin lymphoma, colorectal cancer, papillary thyroid carcinoma, non-small-cell lung carcinoma, and adenocarcinoma of the lung, while somatic *EGFR* mutations are observed in lung cancers[11]. Additionally, *PIK3CA* mutations are more frequent in breast and colorectal cancer[12]. *FOXL2* mutations are commonly seen in granulosa cell tumors, and *TP53* mutations are detected in almost all cancer types[9]. Additionally, recurrent alterations have been found in specific tumor types and across different cancers. These mutations occur at "***hotspots***" or places in the DNA sequence that are both vulnerable to mutagenesis and of significant impact on the protein function in a way that alters cellular growth and life cycle.

Knowing that a patient's tumor has developed one or more of these hotspot mutations can help the physician develop a personalized treatment plan while monitoring disease response and potential drug resistance. For example, in metastatic melanoma patients found to harbor a specific somatic *BRAF* mutation (V600E) in their tumor, treatment with BRAF inhibitors dabrafenib, trametinib and vemurafenib either alone or in combination is often recommended[13]. In addition, the EGFR inhibitors cetuximab and panitumumab are most useful in the lung cancer patients whose tumors are *KRAS* wild type (which means they do not have a mutation in the *KRAS* gene) and whose tumors express EGFR protein. Additionally, several large clinical trials have also shown that the EGFR tyrosine kinase inhibitors (TKIs) afatinib and erlotinib are useful only for treating patients whose tumors are found to carry EGFR kinase domain mutations[14].

Recent studies have shown the feasibility of using liquid biopsies to monitor tumor dynamics. Several studies have shown that the somatic mutations identified through a liquid biopsy of a patient's blood correlate with those found in the tumor specimen obtained either through biopsy or surgical resection[15,16]. Utilizing ctDNA to identify somatic mutations in tumors has been shown to correlate with clinical and radiologic outcomes for a patient as well as to predict the overall survival of the patient in some cases[12,13,15-17]. Additionally, several studies have shown that the reappearance or rising levels of ctDNA can be seen months before clinical signs or symptoms or imaging changes become apparent. Thus, serial evaluation of ctDNA has been shown to be helpful in tracking disease progression, as well as in identifying the appearance of additional somatic mutations, which may be associated with drug sensitivity and resistance, in several different cancer types[18]. A recent study by Perrone et al. (2014) showed promising results for the application of ctDNA mutation analysis as a screening tool for individuals at high risk of developing colorectal cancer.[40]

ctDNA has now been used as a biomarker in an increasing number of cancer types, including lymphoma, melanoma, GIST, thyroid cancer, breast cancer, colon, and lung cancer monitoring for common mutations, (e.g., those found in BRAF, EGFR , KRAS, PIK3CA, TP53, KIT, PDGFRA), and has informed prognosis and treatment decisions (Gonzalez-Cao et al. 2015; Kang et al. 2015; Lubitz et al. 2016; Piotrowska et al. 2015; Roschewski et al. 2015; Schiavon et al. 2015; Sefrioui et al. 2015; Spindler et al. 2015; Xu et al. 2015; Yoo et al. 2014). As a result of these promising initial studies, ctDNA analysis is now being incorporated into several ongoing clinical trials (Polivka et al. 2015). ctDNA analysis is likely to be useful for tumor molecular profiling as it has several potential advantages over traditional tumor biopsy. It is possible that ctDNA analysis will be used to monitor tumors over time in response targeted therapeutics, to monitor the development of resistance, and for the detection of minimal residual disease (Arnedos et al. 2015; Bordi et al. 2015; Diaz and Bardelli 2014; Garcia-Murillas et al. 2015;Saliou et al. 2015; Siravegna et al. 2015; Yoo et al. 2014). Further, the amount and type of ctDNA recovered may be indicative of tumor stage and burden (Diaz and Bardelli 2014; Ocana et al. 2015; Saliou et al. 2015), and thus potentially used for tumor staging. Further, in addition to gene specific mutation detection, ctDNA has been used to detect other tumor-specific genetic alterations, including microsatellite instability, loss of heterozygosity, and epigenetic changes (Aarthy et al. 2015). Importantly, ctDNA may be one day be used in cancer screening and for early detection of disease (Amant et al. 2015; Bianchi et al. 2015).

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Table 2 – Frequency of somatic mutations by genes and cancer types

Cancer type	Gene name	Frequency of somatic mutations
Lung cancer	*BRAF*	1-4% 1% in Non Small Cell Lung Cancer (NSCLC)
	EGFR	29%
	KRAS	17%
	PIK3CA	4%
	TP53	34%

References: COSMIC database (http://cancer.sanger.ac.uk/cosmic) accessed 07/28/2015 [19-25].

OncoNext Liquid™ test

A liquid biopsy, such as GENOMA's **OncoNext Liquid™** test, involves a blood collection (one tubes x10 ml blood). Once collected, the blood sample is centrifuged to separate the plasma containing the cell-free DNA from other components. The cfDNA is then extracted, amplified, and then analyzed for the specific somatic mutations of interest by next-generation sequencing (NGS).
Being able to analyze the liquid biopsy for multiple mutations simultaneously allows the clinician to better understand the tumor profile and adapt treatment appropriately. Blood samples can be drawn from a patient before, during, and/or after cancer treatment, or at regular intervals. Liquid biopsy has the potential for continual monitoring, which is a major advantage in cancer care.

Potential indications for the OncoNext Liquid™ test

OncoNext Liquid™ *Monitor* test is meant for patients who have been **diagnosed with cancer**, in order to:

- **Provide tumor profiling for precision medicine**: The **OncoNext Liquid™** *Monitor* test can provide physicians with valuable information about a patient's tumor profile (somatic mutations present in the tumor), which can be utilized in the development of a personalized treatment plan.
- **Monitor treatment efficacy in patients**. When a patient starts a new treatment, **OncoNext Liquid™** *Monitor* test provides a novel way of reviewing the treatment's effectiveness (e.g. monitoring the presence of mutations prior to and during treatment).
- **Monitor residual disease and/or recurrence in patients with known mutations in the primary tumor**: in instances where patients have undergone a resection of their tumor and/or have gone into disease remission, serial analysis of ctDNA burden utilizing the **OncoNext Liquid™** *Monitor* test, can help check the development of disease reoccurrence or progression.
- **Monitor disease progression and tumor evolution**: While patients are undergoing cancer treatment, oncologists can use the **OncoNext Liquid™** *Monitor* test to check the development of the patient's tumor progression and/or tumor evolution (changes in the type of mutations within a tumor). It is important to evaluate tumor evolution throughout treatment as it can lend information about potential drug sensitivity and resistance.
- **Help the physician explore other options of treatment when the patient is resistant to current therapies**.
- **Provide an alternative method for biopsy** when tissue is difficult to obtain or not available, or when the primary site of metastatic disease is unknown, or when the quantity of tissue obtained in a biopsy sample is limited and traditional molecular genotyping is requested.
- **Provide prognostic information** for some patients.
- **Clinical Trial Matching:** it is an additional feature of the **OncoNext Liquid™** *Monitor* test, which gives patients the option to receive personalized information about clinical trials that may be best suited for him or her, based on their tumor's profile.

OncoNext Liquid™ test technology

The **OncoNext Liquid™** test is designed for the detection of hotspot somatic mutations in a set of **11 driver genes** involved primarily in **lung** cancer: **ALK, BRAF, EGFR, ERBB2, KRAS, MAP2K1, MET, NRAS, PIK3CA, ROS1, TP53**. The assay requires a blood sample (plasma) for cell free DNA isolation, which is used for PCR amplification of both the wild type and mutant DNA. The mutant DNA is sequenced on Illumina's next-generation sequencing platforms.

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Reported Genes and Mutations

GENOMA's **OncoNext Liquid™** test detects frequently occurring **hotspot mutations** in **11** cancer driver genes (**Table 3**), implicated in lung cancer.
The gene content was carefully selected to include content cited by industry organizations such as the National Comprehensive Cancer Network (NCCN)[59] and the European Society for Medical Oncology (ESMO)[60]. These genes and gene regions include single nucleotide variants (SNV) and insertions and deletions (indels) that have demonstrated involvement in tumors.
Research studies and clinical trials have shown that these mutations may reflect tumor burden, treatment response and resistance, and disease prognosis. The majority of somatic mutations are activating mutations, meaning they increase the activity of the protein coded by the gene, which leads to continuous release of growth signals, increased cell proliferation and may contribute to tumor formation.

Table 3A: Genes investigated, targeted therapies and role in cancer

Gene	Targeted Therapies	Role in Cancer
ALK	LDK378, Crizotnib, X-396	The anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase that is aberrant in a variety of cancers. For example ALK fusions are found in anaplastic large cell lymphoma, colorectal cancer, inflammatory myofibroblastic tumor non-small cell lung cancer, and ovarian cancer. All ALK fusions contain the entire ALK tyrosine kinase domain. ALK fusions and copy number gains have been observed in renal cell carcinoma. Signaling downstream of ALK fusions results in activation of cellular pathways known to be involved in cell growth and cell proliferation.
BRAF	Trametinib, Vemurafenib, Dabrafenib, MEK & RAS inhibitors	BRAF belongs to the RAF family of serine-threonine protein kinases that activate MAPK/ERK signaling to regulate cell division and differentiation. BRAF mutations leading to constitutive kinase activation have been implicated in the pathogenesis of several cancers, including melanoma, non-small cell lung cancer, colorectal cancer, papillary thyroid cancer, and ovarian cancer with incidence ranging from 1 to 42%.
EGFR	Erlotinib, Panitumumab, Cetuximab, Neratinib	The Epidermal Growth Factor Receptor (EGFR) belongs to a family of receptor tyrosine kinases that include EGFR and HER2/ERBB2/NEU. Activating mutations, which occur in 1-5% breast and colorectal cancers, cause misregulation of EGFR tyrosine kinase activity resulting in altered multiple downstream pathways involved in cell survival, and cell proliferation.
ERBB2	Trastuzumab, Lapitinib, Kadcyla, Pertuzumab, MGAH22, AMG 386, LJM 716, Neratiinib, Ganestespib, MM-302	HER2 belongs to a family of receptor tyrosine kinases that includes EGFR, HER2/ERBB2/NEU, and HER3/ERBB3. The gene for HER2 has been found to be genetically amplified/mutated in several human cancers including breast and ovarian cancers. HER2 over expression or activating mutations result in constitutive HER2 tyrosine kinase activity. Activated HER2 then regulates proliferation promoting tumorigenesis and pathogenesis.
KRAS	Trametinib, MEK & RAS inhibitors	KRAS protein is a central mediator of growth factor receptor signaling for cell proliferation, survival, and differentiation. KRAS has been implicated in the pathogenesis of several cancers. Activating mutations within the KRAS gene result in constitutive activation and sustained proliferation signals. KRAS is recurrently mutated in several malignancies including colon cancer, breast cancer, lung cancer, and pancreatic cancer.
MAP2K1		
MET	Cabozantinib, Crizotinib	The MET gene encodes a receptor tyrosine kinase (RTK) belonging to the MET/RON family of RTKs. In the context of malignancy, aberrant signaling through the MET receptor promotes pleiotrophic effects including growth, survival, invasion, migration, angiogenesis and metastasis.The MET receptor and/or its ligand HGF have been reported to be aberrantly activated in many human cancers. Germline mutations in the tyrosine kinase domain of MET occur in 100% of hereditary papillary renal cell carcinoma, and somatic mutations in MET are found in 10–15% of sporadic papillary renal cell carcinoma.
NRAS	Trametinib, MEK & RAS inhibitors	RAS has been implicated in the pathogenesis of several cancers. Activating mutations within the RAS gene result in constitutive activation of the RAS GTPase, even in the absence

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		of growth factor signaling. The result is a sustained proliferation signal within the cell. Specific RAS genes are recurrently mutated in different malignancies. NRAS mutations are particularly common in melanoma, hepatocellular carcinoma, myeloid leukemias, and thyroid carcinoma.
PIK3CA	BKM120, MK-2206, BYL719, BEZ235, GDC-0032, GDC-0941, GDC-0980, Everolimus, CC-122, Temsirolimus, AMG479, CC-223,	PIK3CA is the gene for the catalytic subunit of the phosphoinositide 3-kinase, a lipid kinase that regulates many cellular processes including growth, proliferation, differentiation, motility and survival. Mutant PIK3ca has been implicated in the pathogenesis of several cancers including breast cancer (25%), colon cancer (14%), gastric (9%), lung (4%) and endometrial cancers (23%).
ROS1		
TP53	Therapeutic target	TP53 is a tumor suppressor gene; loss or mutation of TP53 protein may result in genomic instability and excessive cell proliferation. Mutations of TP53 have been reported in approximately 30% of all cancer including 44% of large intestine carcinoma, 18% prostate and 22% of breast cancer.

Table 3B: Genes investigated and type of cancer

GENE	Targeted Therapies	RESISTENCE	Type of cancer
ALK	Alectinib Crizotinib Ceritinib LDK378 X-396	Alectinib Crizotinib ceritinib	Lung, Neuroblastoma, Rhabdomyosarcoma
BRAF	Dabrafenib Vemurafenib Trametinib MEK & RAS inhibitors	Dabrafenib vemurafenib	Melanoma*, Colorectal* Lung, Ovarian, Gastric, Glioma, Thyroid, Pancreas, Prostate
EGFR	Afatinib Axitinib Cetuximab Erlotinib Gefitinib Lapatinib Linifatinib Motesanib Neratinib Panitumumab Pelitinib Ponatinib Sorafenib Sunitinib tivozanib	Afatinib inibitori tirosin kinasici gefitinib erlotinib azd9291	Lung*; Head & Neck, Prostate, Breast, Ovarian
ERBB2	Afatinib AMG 386 Ganestespib Kadcyla Lapatinib LJM 716 MGAH22 MM-302 Neratiinib Trastuzumab		Breast, Lung, Ovarian
KRAS	Trametinib MEK & RAS inibititori		Colorectal*, Gastric, Lung*, Ovarian, Thyroid, Endometrial, Pancreas, Prostate
MAP2K1			
MET	Crizotinib cabozantinib		Lung*, Colorectal, Gastric
NRAS	Trametinib MEK & RAS inibititori		Colorectal*, Lung, Melanoma, Thyroid

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PIK3CA	Alpelisib AMG479 BEZ235 BKM120 BYL719 Buparlisib CC-122 CC-223 Everolimus GDC-0032 GDC-0941 GDC-0980 MK-2206 Temsirolimus		Lung, Breast, Prostate, Colorectal, Ovarian, Head & Neck, Pancreas, Thyroid, endometrial, gastric
ROS1			
TP53			Lung, Melanoma, Ovarian, Colorectal, Breast; Endometrial, Head & Neck, Kidney, Pancreas, Prostate, Thyroid

† Targeted therapy source: Breastcancertrials.org (updated 12/16). Select targeted therapies shown; not comprehensive of all agents in development. Includes investigational agents as well as commercialized drugs.

Possible outcomes

GENOMA's **OncoNext Liquid™** test reports on the absence or presence of each of the hotspot mutations above 2 mutant DNA copies per patient plasma sample. Input DNA is the total amount of cfDNA from the provided patient plasma sample used in the assay. The total number of detected mutant copies of ctDNA are reported. Input and mutant DNA content are variable. Mutant DNA percentage is also reported relative to input, with reference to limit of detection (LOD). Personalized interpretation of the result based on the individual's clinical history is provided. Optional clinical trial matching based on the results may be requested for patients with advanced disease tested via **OncoNext Liquid™** Monitor analysis.

Clinical utility

In a large study of subjects with various cancers, Bettegowda et al (2014) showed that ctDNA was detected in more than 75% of patients with advanced (metastatic) disease (pancreatic, ovarian, colorectal, bladder, gastroesophageal, breast, melanoma, hepatocellular, and head and neck cancers) and less than 50% of primary (Stage 1) cancers (brain, renal, prostate, or thyroid cancers)[26]. In a separate group of 206 metastatic colorectal cancer patients, the authors showed high sensitivity and specificity of ctDNA detection for clinically relevant *KRAS* gene mutations (87.2% and 99.2%, respectively).

Kidess et al (2015) detected somatic mutation in 68% of colorectal cancer patients (n=38) in a 46 mutations panel which included *BRAF, KRAS, EGFR* and *PIK3CA* [8]. 54% early (Stage I-III) and 93% advanced (Stage IV) cancer patients showed presence of mutations. Fifty percent patients had *KRAS*, 16% *PIK3CA* and 8% had *BRAF* mutations. No *EGFR* mutations were detected in the study. These data were in concordance with mutations identified in the tumors. In patients undergoing liver metastatectomy, ctDNA levels predicted tumor recurrence earlier than carcinoembryonic antigen (CEA) value or imaging. Four of the healthy individuals (n=47) also showed signals at or near the limit of detection of ctDNA.

In one of the largest multicentric clinical trial (CORRECT), effect of a multikinase inhibitor, regorafenib was evaluated by assessing ctDNA levels in metastatic colorectal cancer patients[18]. Mutation analysis was performed in a total of 760 patients which included 505 regorafenib treated and 255 placebo. Comparison of patient matched archived tumor specimen and fresh plasma showed concordant mutations ranging from 76% - 97% for the three genes. *KRAS* mutations were identified in 69%, *PIK3CA* mutations in 84% and *BRAF* mutations in 3% of the patients. In the regorafenib treated group, patients with *KRAS* mutations showed a significantly reduced progression free and overall survival as compared to the placebo. Interestingly, in the placebo group, patients with higher ctDNA had poor overall and progression free survival. The study supports the use of ctDNA to establish tumor genotypes at the time of treatment.

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Oshiro et al (2015) reported that *PIK3CA* mutant ctDNA positive patients had significantly lower recurrence free survival (RFS) than patients with negative ctDNA in a study conducted on stage I to III breast cancer patients (n=313)[27]. The patients with higher counts of *PIK3CA* mutant ctDNA (>29 alleles) showed significant lower recurrence free and overall survival. Additionally, Beaver et al (2014) detected *PIK3CA* mutations in 12 out of 29 breast cancer patients (stage I-III patients). One patient with persistent ctDNA following initial treatment developed clinically apparent metastasis 23 months later[28].

Dawson et al (2013) detected ctDNA in nearly 97% of the metastatic breast cancer patients, and showed a higher sensitivity and specificity of ctDNA measurements in detecting tumor as compared to the CTCs and CA15-3 levels[29]. *PIK3CA* and *TP53* point mutations correlated with disease and the patients with higher level of ctDNA showed poor prognosis.

Janku et al (2015) tested 21 mutations in *BRAF, EGFR*, *KRAS* and *PIK3CA* in 157 patients with advanced cancers (including colorectal, melanoma, NSCLC, appendiceal cancer, ovarian and uterine cancers) who progressed on systemic therapy[17]. The authors found significant similarity in the mutations detected in archival tissue and mutations identified in ctDNA. The also found that forty-one patients with more than 1% of KRAS mutant cfDNA had a shorter median survival compared to the 20 patients with ≤ 1% of KRAS mutant DNA (4.8 vs. 7.3 months, p = 0.008). Similarly, 67 patients with >1% of mutant cfDNA (*BRAF, EGFR, KRAS,* or *PIK3CA*) had a shorter median survival compared to 33 patients with ≤ 1% of mutant cfDNA (5.5 vs. 9.8 months, p = 0.001).

Obtaining enough tissue to perform histology on a biopsy may be difficult in advanced pancreatobiliary carcinoma patients. Zill et al (2015) used ctDNA measurements to analyze mutations in a set of 54 genes. *KRAS* and *TP53* were the most commonly mutated genes, with *APC*, *SMAD4*, *GNAS*, *FBXW7*, and *BRAF* also being recurrently mutated in the patient ctDNA[16]. Across these five genes (*KRAS*, *TP53*, *APC*, *FBXW7*, and *SMAD4)* the average sensitivity was 92.3%, specificity was 100%, and average diagnostic accuracy was 97.7%. The authors also identified actionable mutations during the follow-up of the patients which otherwise was undetected due to the failure of initial tissue biopsy.

The growing evidence in the field of liquid biopsy suggests that these types of biomarker analyses can be applied to patients with multiple different types of cancers[8, 9, 16, 17, 26, 27, 29-35]. More and more, ongoing clinical trials are now incorporating liquid biopsies to evaluate therapy response (www.clinicaltrials.gov).

Limitations and warnings

GENOMA's OncoNext Liquid™ (Monitor) test is a plasma-based hotspot mutation panel to aid clinicians in the identification of plasma mutation tumor burden, monitoring of cancer patients, treatment planning and preventative surveillance of high risk cancer populations for early detection of disease.
Cancer is heterogeneous disease that can occur as a result of somatic mutations in various driver genes. OncoNext Liquid™ (Monitor or Scan) identifies somatic cancer derived hotspot mutations in 50 cancer driver genes. This test is not meant to diagnose cancer, and is only meant to screen for a possible malignancy as an adjunct to other medical examinations and interventions. It will not detect all cancers, and has not been designed to find very small tumors. No test can replace a physician's examination, imaging studies, and tissue biopsies as the gold-standard for cancer diagnosis. It is possible that mutations in these or other genes not tested in GENOMA's OncoNext Liquid™ (Monitor or Scan) test may be involved in the patient's disease. Therefore, a negative test result, where no mutations are detected, does not eliminate involvement of other genes and/or mutations. Furthermore, a positive test result needs to be interpreted in the context of individual's clinical history including stage of disease, imaging results, therapeutic details, and other laboratory data.
Results could be misinterpreted if clinical information provided is inaccurate or incomplete. Improper blood sampling and handling could result in error. Genetic counseling or medical consultation is recommended for the individual tested.

TECHNICAL INFORMATION

Assay Method

OncoNext Liquid™ (Monitor) test identifies hotspot somatic mutations (**table 4**) in a set of 11 genes

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commonly involved in, but not limited to, breast, lung, colorectal, and ovarian cancers and melanoma. Cell free DNA containing circulating tumor DNA is isolated from plasma, quantitated and PCR amplified. The mutant DNA pool is then PCR amplified for sequencing on next-generation sequencing platforms.

Sequencing

Libraries from plasma cfDNA, were sequenced on a NGS sequencer.

Range

OncoNext Liquid™ (Monitor) test reports on the absence or presence of each of the hotspot somatic mutations with > 2 mutant DNA copies per patient plasma sample. Input DNA is the total amount of cfDNA from the provided patient plasma sample used in the assay. The total number of detected mutant copies of ctDNA are reported. Input and mutant DNA content are variable. Mutant DNA % is also reported relative to input, with reference to limit of detection (LOD). Databases queried include Catalogue of Somatic Mutations in Cancer (COSMIC), The Cancer Genome Atlas (TCGA), cBioPortal, National Center of Biotechnology Information (NCBI), locus specific databases and other public databases. Personalized interpretation of the result based on the individual's clinical history is provided. Optional clinical trial matching based on the results for patients with advanced disease may be requested.

Expected Values

0, 1 or more variants

Technical limitations

Gene amplifications, translocations, and insertions or deletions over 25 bases in length are not detectable by this assay. Variants predicted to be non-deleterious (such as synonymous coding changes and common population variants) are not reported.
In validation studies, the analytical sensitivity and specificity of the targeted cancer gene assay were > 99% and > 99.9%, respectively. These may be lower for structural alterations and vary depending on the quality of the specimen. Next generation sequencing approaches may provide incorrect sequence or mutational data due to insufficient coverage in specific regions of the genome, inability to distinguish highly related human sequences, and sequencing errors.
The analysis of sequence specific alterations can also be hampered by three aspects related to the tumor DNA. First, the quality of tumor DNA obtained; second, the quantity of DNA obtained can be very low, limiting the amount of DNA molecules that can be successfully analyzed by next generation sequencing. Third, the purity of tumor DNA can be a factor, as a significant portion of the DNA analyzed in the tumor sample may be derived from contaminating normal tissues. These three aspects can reduce the chance of detecting somatic sequence and copy number alterations and rearrangements. Genetic alterations are defined as clinically significant based on published literature and other evidence. Literature references are not comprehensive and there may be other studies that relate to the test results. This test, meant to identify somatic mutations, is not intended to detect the presence or absence of germline mutations.

Target Coverage

Coverage is the number of times a region is sequenced (the number of reads) within a single run. In general, the deeper the coverage of a targeted region, the more sensitive and reliable the assay is. For variant calling, 25.000x coverage is required for reliable detection of mutations occurring at frequencies as low as 0.1%. To pass quality control (QC) metrics for the **OncoNext Liquid™** test, samples should yield > 25.000x coverage on > 93.5% of bases targeted by the assay. Libraries generated from analytical cfDNA and clinical cfDNA performed with > 99.8% of amplicons yielding coverage ≥25.000x (**Table 4**).

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Mutant Allele Fraction (MAF)

The mutant allele fraction is the frequency of the mutant allele identified in the sample and is reported for base substitutions, insertions and deletions.

Performance specifications

Mutant Allele Frequency (MAF) / Tumor Fraction	Sensitivity	Positive Predictive Value (PPV)
≥0.1%	99% (97.2%-100%)*	99.9% (99.4%-100%)*

*95% Confidence Interval

Disclaimer

Results presented in this report are intended for use solely by a qualified health care professional. Any diagnosis, counseling, or treatment determination made as a result of data presented in the report should be made by a qualified health care professional in conjunction with other individual patient health information, including clinical presentation and other test reports. Information contained within the report is current as of the report date; a qualified health professional should reassess these data as relevant literature becomes available.

Table 4: Hotspot mutations investigated with OncoNext Liquid™ Lung

GENE	MUTATION	COSMIC ID	Chromosome	HG19_coordinates (start-end)	
ALK	p.R1275L	COSM28060	chr2	29432663	29432664
ALK	p.R1275Q	COSM28056	chr2	29432663	29432664
ALK	p.F1245L	COSM28062	chr2	29436857	29436858
ALK	p.F1245L	COSM28493	chr2	29436857	29436858
ALK	p.F1245C	COSM28500	chr2	29436858	29436859
ALK	p.F1245I	COSM28492	chr2	29436859	29436860
ALK	p.F1245V	COSM28499	chr2	29436859	29436860
ALK	p.L1196Q	COSM1169447	chr2	29443629	29443630
ALK	p.L1196M	COSM99137	chr2	29443630	29443631
ALK	p.V1180L	COSM4381101	chr2	29443678	29443679
ALK	p.F1174L	COSM28055	chr2	29443694	29443695
ALK	p.F1174L	COSM28061	chr2	29443694	29443695
ALK	p.F1174C	COSM28059	chr2	29443695	29443696
ALK	p.F1174S	COSM53063	chr2	29443695	29443696
ALK	p.F1174I	COSM28491	chr2	29443696	29443697
ALK	p.F1174V	COSM28054	chr2	29443696	29443697
ALK	p.F1174L	COSM28057	chr2	29443696	29443697
ALK	p.I1171N	COSM1169448	chr2	29445211	29445213
ALK	p.I1171N	COSM28498	chr2	29445212	29445213
ALK	p.I1171T	COSM4381100	chr2	29445212	29445213
ALK	p.C1156Y	COSM99136	chr2	29445257	29445258
ALK	p.L1152P	COSM1407659	chr2	29445269	29445270
ALK	p.L1152R	COSM97185	chr2	29445269	29445270
ALK	p.T1151_L1152insT	COSM144252	chr2	29445271	29445271
ALK	p.G1128A	COSM98475	chr2	29445449	29445450
BRAF	p.V600E	COSM476	chr7	140453135	140453136
BRAF	p.G469V	COSM459	chr7	140481401	140481402
BRAF	p.G466V	COSM451	chr7	140481410	140481411
BRAF	p.Y472C	COSM1133046	chr7	140481392	140481393

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BRAF	p.L597V	COSM470	chr7	140453145	140453146
BRAF	p.G469A	COSM460	chr7	140481401	140481402
BRAF	p.Gly469Leu	COSM1548505	chr7	140481401	140481403
EGFR	p.E709K	COSM12988	chr7	55241676	55241677
EGFR	p.E709A	COSM13427	chr7	55241677	55241678
EGFR	p.G719C	COSM6253	chr7	55241706	55241707
EGFR	p.G719S	COSM6252	chr7	55241706	55241707
EGFR	p.G719A	COSM6239	chr7	55241707	55241708
EGFR	p.K745_E746insIPVAIK	COSM51504	chr7	55242464	55242464
EGFR	p.E746_A750delELREA	COSM6223	chr7	55242464	55242479
EGFR	p.E746_A750delELREA	COSM6225	chr7	55242465	55242480
EGFR	p.E746_T751A	COSM12678	chr7	55242466	55242481
EGFR	p.E746_S752V	COSM12384	chr7	55242466	55242485
EGFR	p.L747_E749delLRE	COSM6218	chr7	55242468	55242477
EGFR	p.L747_A750P	COSM12382	chr7	55242468	55242478
EGFR	p.L747_T751P	COSM12383	chr7	55242468	55242481
EGFR	p.L747_S752delLREATS	COSM6255	chr7	55242468	55242486
EGFR	p.L747_T751delLREAT	COSM12369	chr7	55242469	55242484
EGFR	p.L747_P753S	COSM12370	chr7	55242469	55242487
EGFR	p.S768I	COSM6241	chr7	55249004	55249005
EGFR	SYS_ERR	SYS_ERR	chr7	55249005	55249005
EGFR	p.V769_D770insASV	COSM12376	chr7	55249009	55249009
EGFR	p.D770_N771insSVD	COSM13428	chr7	55249013	55249013
EGFR	p.H773_V774insH	COSM12377	chr7	55249021	55249021
EGFR	p.H773_V774insNPH	COSM12381	chr7	55249021	55249021
EGFR	p.T790M	COSM6240	chr7	55249070	55249071
EGFR	p.C797S	COSM2741500	chr7	55249091	55249092
EGFR	p.E709_T710>D	COSM51525	chr7	55241678	55241681
EGFR	p.E709_T710>A	COSM85796	chr7	55241677	55241680
EGFR	p.E709_T710>G	COSM48981	chr7	55241677	55241681
EGFR	p.E709H	COSM12428	chr7	55241676	55241679
EGFR	p.E709G	COSM13009	chr7	55241677	55241678
EGFR	p.E709V	COSM12371	chr7	55241677	55241678
EGFR	p.G719D	COSM18425	chr7	55241707	55241708
EGFR	p.H835L	COSM6227	chr7	55259445	55259446
EGFR	SYS_ERR	SYS_ERR	chr7	55259445	55259447
EGFR	p.P848L	COSM22943	chr7	55259484	55259485
EGFR	SYS_ERR	SYS_ERR	chr7	55259484	55259485
EGFR	SYS_ERR	SYS_ERR	chr7	55259484	55259485
EGFR	SYS_ERR	SYS_ERR	chr7	55259484	55259485
EGFR	p.L858R	COSM6224	chr7	55259514	55259515
EGFR	p.L861Q	COSM6213	chr7	55259523	55259524
ERBB2	p.A775_G776insYVMA	COSM20959	chr17	37880995	37880995
KRAS	p.Q61H	COSM554	chr12	25380274	25380275
KRAS	p.Q61R	COSM552	chr12	25380275	25380276
KRAS	p.Q61L	COSM553	chr12	25380275	25380276
KRAS	p.G13D	COSM532	chr12	25398280	25398281
KRAS	p.G13C	COSM527	chr12	25398281	25398282
KRAS	p.G12V	COSM520	chr12	25398283	25398284
KRAS	p.G12D	COSM521	chr12	25398283	25398284
KRAS	p.G12A	COSM522	chr12	25398283	25398284
KRAS	p.G12F	COSM512	chr12	25398283	25398285
KRAS	p.G12C	COSM516	chr12	25398284	25398285
KRAS	p.G12S	COSM517	chr12	25398284	25398285

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KRAS	p.G12R	COSM518	chr12	25398284	25398285
MAP2K1	p.F53I	COSM3503329	chr15	66727440	66727441
MAP2K1	p.F53L	COSM555604	chr15	66727440	66727441
MAP2K1	p.F53L	COSM1562837	chr15	66727441	66727442
MAP2K1	p.F53L	COSM1725008	chr15	66727442	66727443
MAP2K1	p.K57Q	OM3155	chr15	66727452	66727453
MAP2K1	p.Q56P	COSM1235481	chr15	66727450	66727451
MAP2K1	p.K57T	COSM4756761	chr15	66727453	66727454
MAP2K1	p.Lys57Asn	COSM1235478	chr15	66727454	66727455
MAP2K1	p.P124S	COSM235614	chr15	66729161	66729162
MAP2K1	p.P124Q	COSM1167912	chr15	66729162	66729163
MAP2K1	p.P124L	COSM1315861	chr15	66729162	66729163
MAP2K1	p.E203K	COSM232755	chr15	66774130	66774131
MAP2K1	p.E203V	COSM3386991	chr15	66774131	66774132
MET	p.T1010I	COSM707	chr7	116411989	116411990
MET	p.Y1021N	COSM48564	chr7	116412021	116412022
MET	p.Y1021F	COSM339515	chr7	116412022	116412023
MET	SYS_ERR	SYS_ERR	chr7	116412043	116412045
MET	NA	COSM24687	chr7	116412043	116412044
MET	NA	COSM29633	chr7	116412043	116412044
MET	NA	COSM35468	chr7	116412044	116412045
MET	p.H1112Y	COSM696	chr7	116417462	116417463
MET	p.H1112L	COSM698	chr7	116417463	116417464
MET	p.H1112R	COSM703	chr7	116417463	116417464
MET	p.Y1248H	COSM690	chr7	116423412	116423413
MET	p.Y1248C	COSM699	chr7	116423413	116423414
MET	p.Y1253N	COSM1447477	chr7	116423427	116423428
MET	p.Y1253H	COSM598581	chr7	116423427	116423428
MET	p.Y1253D	COSM700	chr7	116423427	116423428
MET	p.M1268V	COSM1568673	chr7	116423472	116423473
MET	p.M1268T	COSM691	chr7	116423473	116423474
MET	p.M1268I	COSM694	chr7	116423474	116423475
NRAS	p.Q61L	COSM583	chr1	115256528	115256529
NRAS	p.Q61K	NA	chr1	115256529	115256530
NRAS	p.A59T	NA	chr1	115256535	115256536
NRAS	p.G13V	COSM572	chr1	115258742	115258744
NRAS	p.G13D	COSM573	chr1	115258743	115258744
NRAS	SYS_ERR	SYS_ERR	chr1	115258746	115258747
NRAS	p.G13Y	COSM568	chr1	115258743	115258745
NRAS	p.G13V	COSM574	chr1	115258743	115258744
NRAS	p.G13A	COSM575	chr1	115258743	115258744
NRAS	p.G13N	COSM24668	chr1	115258743	115258745
NRAS	p.G13R	COSM569	chr1	115258744	115258745
NRAS	p.G13C	COSM570	chr1	115258744	115258745
NRAS	p.G13S	COSM571	chr1	115258744	115258745
NRAS	p.G12E	COSM144577	chr1	115258745	115258747
NRAS	p.G12D	COSM564	chr1	115258746	115258747
NRAS	p.G12P	COSM559	chr1	115258746	115258748
NRAS	p.G12Y	COSM560	chr1	115258746	115258748
NRAS	p.G12A	COSM565	chr1	115258746	115258747
NRAS	p.G12V	COSM566	chr1	115258746	115258747
NRAS	p.G12N	COSM12723	chr1	115258746	115258748
NRAS	p.G12R	COSM561	chr1	115258747	115258748
NRAS	p.G12C	COSM562	chr1	115258747	115258748

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NRAS	p.G12S	COSM563	chr1	115258747	115258748
PIK3CA	p.E542K	COSM760	chr3	178936081	178936082
PIK3CA	p.E545K	COSM763	chr3	178936090	178936091
PIK3CA	p.H1047R	COSM775	chr3	178952084	178952085
ROS1	p.L1951M	COSM1072521	chr6	117641119	117641120
TP53	p.R337L	COSM11411	chr17	7574016	7574017
TP53	p.R283P	COSM10743	chr17	7577089	7577090
TP53	p.R282W	COSM10704	chr17	7577093	7577094
TP53	p.R280I	COSM11287	chr17	7577098	7577099
TP53	p.C277F	COSM10749	chr17	7577107	7577108
TP53	p.R273H	COSM10660	chr17	7577119	7577120
TP53	p.R273L	COSM10779	chr17	7577119	7577120
TP53	p.R273P	COSM43896	chr17	7577119	7577120
TP53	p.R273C	COSM10659	chr17	7577120	7577121
TP53	p.R249S	COSM10785	chr17	7577533	7577534
TP53	p.R249S	COSM10817	chr17	7577533	7577534
TP53	p.R249M	COSM43871	chr17	7577534	7577535
TP53	p.R248Q	COSM10662	chr17	7577537	7577538
TP53	p.R248L	COSM6549	chr17	7577537	7577538
TP53	p.R248W	COSM10656	chr17	7577538	7577539
TP53	p.G245V	COSM11196	chr17	7577546	7577547
TP53	p.G245C	COSM11081	chr17	7577547	7577548
TP53	p.C242F	COSM10810	chr17	7577555	7577556
TP53	p.M237I	COSM10834	chr17	7577569	7577570
TP53	p.Y234C	COSM10725	chr17	7577579	7577580
TP53	p.Y220C	COSM10758	chr17	7578189	7578190
TP53	p.H214R	COSM43687	chr17	7578207	7578208
TP53	p.Y205C	COSM43947	chr17	7578234	7578235
TP53	p.H179R	COSM10889	chr17	7578393	7578394
TP53	p.C176F	COSM10645	chr17	7578402	7578403
TP53	p.C176Y	COSM10687	chr17	7578402	7578403
TP53	p.R175H	COSM10648	chr17	7578405	7578406
TP53	p.V173L	COSM43559	chr17	7578412	7578413
TP53	p.Y163C	COSM10808	chr17	7578441	7578442
TP53	p.A159V	COSM11148	chr17	7578453	7578454
TP53	p.R158L	COSM10714	chr17	7578456	7578457
TP53	p.V157F	COSM10670	chr17	7578460	7578461
TP53	p.G154V	COSM6815	chr17	7578468	7578469
TP53	p.T125T	COSM45940	chr17	7579311	7579312

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