Diagnostic Potential of Platelets Derived Selected Tumor Biomarkers in Hepatocellular Carcinoma

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(Supervisor)

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Dedicated to

My Beloved Family Especially My Brother

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All grace and gratitude for the One Who doesn't leave me for the blink of an eye, Who listens to all my whims and woes and walks all roads with me; to the Almighty, the creator the Exalted.

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Sidra Asghar

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LIST OF ACRONYMS

Abbreviation	Full form
%	Percentage
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C virus
NK cells	Natural Killer cells
PI3K	Phosphatidylinositol 3-kinase
RNA	Ribose nucleic acid
WHO	World Health Organization
TEP	Tumor educated Platelet
CTCs	Circulating Tumor cells
cfRNA	Cell free RNA
HBV	Hepatitis B virus

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ABSTRACT

Cancer is a word attributed to a group of diseases which have the characteristic abnormal growth of cells and exhibit the potential to invade the surrounding. Liver cancer is considered to be the sixth in number when ranking the deadliest cancers worldwide. Liver cancer can be of several types depending upon the place of its origin. Among all the types of liver cancer, hepatocellular carcinoma (HCC) is the most common type. The number of HCC cases per year are reported to be increasing due to several contributing factors. The main causative factor for its increasing prevalence is the late diagnosis of the disease at its advance stage.

The methods used for detection of liver cancer include CT scan and tissue biopsy. The main drawback of these methods is that patient goes for these procedures when the disease has already progressed due to their high cost and invasive nature. This limitation further contributes in the prevalence of the disease. To overcome these limitations there is a need to shift towards a less invasive procedure which is both cost effective and has accuracy i.e. liquid biopsy. Liquid biopsy is currently approved for clinical practice for many cancers i.e. NSCLC (Non-Small Cell Lung Cancer)

The aim of the study was to provide basis for the introduction of a non-invasive and effective procedure for HCC diagnosis. Platelets are thought to be the potential candidates for designing liquid biopsy leading to early HCC diagnosis. Platelets are enucleated type of cells found in blood which originate from megakaryocytes. They are very well known for maintaining homeostasis and the initiation of wound healing. Recently, an interaction of tumor cell and platelets has been reported via the transfer of tumor associated molecules.

This is often referred to as education of platelets. Tumor associated biomolecules activate the platelets in a specific way and cause the splicing of pre-mRNA in a unique manner. This specific mRNA repertoire, hence, has the capability to serve as a basis for diagnosis of cancer.

In the present study mRNA repertoire of the platelets from HCC patients as compared to control was observed. Samples from the HCC patients were obtained from Holy family Hospital Islamabad. For the given purpose TGF-β, NF-κB, VEGF, AKT and PI3K were selected to serve as potential biomarkers. The selected genes are well known for playing role in tumor growth and dissemination. Transcriptional analysis of the selected genes depicted that in the platelets from HCC patients, the expression level of TGF-β, NFκβ, and VEGF was significantly increased by 2.48, 2.35 and 2.78 folds respectively. Whereas, a decrease of 0.6 and 0.65 folds was observed in AKT and PI3K respectively as compared to control.

Significant alteration in mRNA levels of the selected biomarkers depicts their potential to be used as possible biomarkers for early diagnosis of HCC. These results in future could be utilized to develop a noninvasive procedure for early detection of HCC with high specificity.

Chapter 1

INTRODUCTION

Primary liver cancer is presented as sixth most commonly occurring primary malignancy worldwide (Valery et al., 2018). It is also considered to be the reason for majority of the deaths attributed to cancers, second only to lung cancer (J Ferlay et al., 2015). Hepatocellular carcinoma (HCC) is the most prevalent type of liver cancer except for some parts of South East Asia (Shin et al., 2010). Numerous risk factors contribute to the development of HCC which include diabetes, smoking, aflatoxin, obesity, chronic infection by Hepatitis B (HBV) virus or Hepatitis C (HCV) and cirrhosis (Bosetti, Turati, La Vecchia, & gastroenterology, 2014; Xia, Jiang, & Peng, 2015). The annual chance of a patient with cirrhosis eventually developing HCC ranges between 1% to 4% (El-Serag, 2012). Another major reason for the high prevalence rate of HCC is the late diagnosis. Patients are usually diagnosed when the disease has progressed to such a stage that the patient is left with very little to zero treatment options.

According to a study out of the total 782, 451 cases reported of HCC globally in 2012, majority of the cases i.e. 83% belonged to the low- and middle-income countries (Ferlay et al., 2013). Although the incidence rate of HCC is lower in high-income countries yet the mortality rate due to Liver cancer has increased as observed in the recent years in USA (Bertuccio et al., 2017). This rate is expected to rise in the next two decades. Pakistan also has an equally moderate to high rate of HCC prevalence in comparison to the rest of the world (S. A. Ali, R. M. Donahue, H. Qureshi, & S. H. J. I. j. o. i. d. Vermund, 2009).

Moreover, an increase in the rate is expected within the near future due to lack of available facilities.

Studies on HCC reveal that around 82.4% of the patients diagnosed with HCC die within the first 5 years post diagnosis (Su, Kim, & Jain, 2018). The main reason for the grim prognosis of HCC are ineffective tools for early detection and limited options for treatment as the disease has high reoccurrence rate.

Current available diagnosis options include tissue biopsy. For cancer study biopsy is done on patient to obtain the tumor tissue. This tissue is further analyzed to obtain the genetic alterations that are either directly or indirectly associated to tumor development (Coppedè, Lopomo, Spisni, & Migliore, 2014; Herman & Baylin, 2003; Ozen et al., 2013; Sharma, Kelly, & Jones, 2010; Yoo & Jones, 2006). Although many consider this method to be a gold standard in cancer diagnosis yet the information obtained after analyzing the tumor is limited making the person unable to determine the heterogeneity of the disease (Awad et al., 2013; Bardelli & Pantel, 2017; Siravegna et al., 2015).

Another approach for early detection of HCC under consideration is liquid biopsy. Advancements in this field hold great promises in improvement of the early diagnosis and increasing the survival rate ultimately. Several studies have evaluated the molecular signatures associated with liver cancer i.e. methylation, other genetic alterations, RNA expression in circulating biomarkers (CB) and Circulating tumor cells (CTCs). The tumor sources that can be exploited for their use in liquid biopsy include cell free DNA, exosomes, CTC and platelets (Su et al., 2018).

These alterations can serve as markers for cancer. Their identification through liquid biopsy should permit not only ambiguous screening but also detection and characterization of the tumor at early stage. Liquid biopsy is not currently practiced for HCC as happening in case of breast cancer, lung cancer and colorectal cancer. However, the exciting applications of liquid biopsy in early detection of cancer, accurately predicting the prognosis as well as easy monitoring of the recurrence of HCC hold great promises for its future use for HCC (Su et al., 2018).

All other sources except platelets show limitation of being very low in number in early stages of cancer thus, hampering their use for early cancer detection. Platelets on the other hand have no such limitation as they are readily available to be used for diagnosis due to their relative abundance in blood. They exist in range of 200 to 500 million per ml of blood. These are anucleate cells which originally originate from megakaryocyte in the bone marrow (Gazzaniga & Ottini, 2001). Platelets carryout various important functions of the body including vessel remodeling, immunity and inflammation.

Apart from their role in performing normal body functions these platelets also play a part in cancer progression. They aid in progression via helping the tumor cell in metastasizing to distant areas of the body (Belloc et al., 1995; Boucharaba et al., 2004). The various paths in which these platelets have been observed to help the cancer cell include intravasation, shielding from the attack of immune cells within the blood, extravasation and ultimately helping the tumor cell seed in a far distant region (Goubran, Stakiw, Radosevic, & Burnouf, 2014; Skog et al., 2008).

It has been proved by different studies performed all over the world that platelets do have the potential to be used as effective biomarkers in early cancer diagnosis. It is also related

to their total count in the blood of the patient as it also varies with the disease and further can assist in progression of the disease (Bottsford-Miller et al., 2015; Cho et al., 2012; Kemona, 2013; Maria Mantur, Koper, Snarska, Sidorska, & Kruszewska-Wnorowska, 2008; Ryningen, Apelseth, Hausken, & Bruserud, 2006; Stone et al., 2012).

Another peculiar property harbored by platelets is their ability to ingest the foreign material secreted by the tumor itself. This can include both the nucleotides as well as the proteins. This foreign material released by the tumor is when ingested by the platelets either by vesicle dependent or independent manner, it causes certain change of events in platelets causing them to undergo peculiar changes. Platelets in such a state is known as being educated by the tumor (Best et al., 2015; Nilsson et al., 2011).

The mRNA repertoire of platelets gets changed by the collective effect of the ingestion of foreign materials and direct interaction with tumor. This highly dynamic mRNA repertoire can thus be exploited for its use as a biomarker in early cancer detection.

All the above stated evidences have laid to the foundation of the present study entitled "Diagnostic Potential of Platelets Derived Selected Tumor Biomarkers in Hepatocellular Carcinoma". As far as I know this is the only study of its kind where we are focusing on exploring the mRNA profile of the platelets extracted from HCC patients. These would further help in predicting the changes which a platelet undergoes while interacting with tumor hence assisting in prediction of HCC in the long run. We, therefore, have selected TGFb, NFkB, VEGF, AKT and PI3K to study their potential to be used as biomarkers for early diagnosis of HCC as all these genes are very well-known for assisting in cell proliferation, tumor metastasis and cancer progression in HCC. This early diagnosis of

HCC would not only be cost effective but would assist in decreasing the HCC prevalence rate overall.

Chapter 2

LITERATURE REVIEW

"Cancer" is basically a generic term. It is used for a group of diseases which are characterized by the abnormal cell growth. These cells also have the potential to invade the adjoining tissues and organs causing their spread. Globally cancer was considered as the second most common cause for deaths in 2015 with 8.8 million deaths, first being the cardiovascular diseases (H. Wang et al., 2016). Cancer burden globally was high with approximately 14 million cases reported. According to an estimation this number will increase in two decades to 22 million (Ghouri, Mian, & Rowe, 2017). This high death rate due to cancer proves that the war with cancer is not over despite of the vast research done on it. Although recent developments in finding new treatments such as immunotherapy are raising expectations but due their high cost they cannot be made available in the third world countries (Hanahan, 2014; Horton & Gauvreau, 2015; Lowy & Collins, 2016).

The uneven distribution of cancer treatment among the developed and underdeveloped countries has raised questions (Levit, Balogh, Nass, & Ganz, 2013). A clear difference is seen in survival rate of cancer patients from developed countries as compared to the underdeveloped regions mainly due to lack of access to the specialized care and effective treatment (Coleman, 2014; Stringhini et al., 2012; Stringhini et al., 2010). This lack of effort for reducing cancer in less privileged countries (Vineis & Wild, 2014) is causing awareness about the "cancer divide" which is having worse outcomes and has become a matter of concern for the International health community (Hanahan, 2014; Horton & Gauvreau, 2015; Vineis & Wild, 2014).

Cancer can occur anywhere in the body but cancer of some areas is more common as compared to the other parts of body. Liver, colorectal, prostate and lung cancer is considered to be more common among men while lung, colorectal, stomach, cervix and breast cancer have high prevalence among women. The incidence rate of liver cancer is specifically is on rise as evident from epidemiology below:

2.1 Epidemiology:

2.1.1 Worldwide prevalence:

Liver cancer has seventh highest rate of incidence globally as 0.8million cases of liver cancer were reported (Ghouri et al., 2017). The most common type of liver cancer i.e. Hepatocellular Carcinoma also known as HCC (Stuver & Trichopoulos, 2008). HCC is considered to be the sixth most commonly found cancer worldwide with respective to occurrence whereas with respect to the mortality rate the case is worse as it is considered to be the second most common cause of death (Jacques Ferlay et al., 2015).The reason for high mortality rate is that the prognosis of HCC is very poor.

HCC has high prevalence rate among the males as compared to females (Ghouri et al., 2017; D Max Parkin, Bray, Ferlay, & Pisani, 2005). Patients are usually of 30-50yr when diagnosed with cancer (Kumar, Abbas, Fausto, & Aster, 2005). Asian countries i.e. Mongolia, China, Africa and Southeast Asia have high predominance rate for HCC (Ghouri et al., 2017). In US prevalence rate is found to be 65% (El-Serag & Mason, 1999; Mittal & El-Serag, 2013). Rate of HCC incidence has been seen to increase according to a report of 2011 (El-Serag & Mason, 1999). Another interesting fact observed was its prevalence among the people with different skin tone. Male and females with darker skin tone were seen to have a higher chance of HCC incidence in comparison to the light skinned

individuals (El-Serag & Mason, 1999). Despite of rise seen in rate of incidence an improvement in survival rate by >60% was observed in a study from year 1975 to 2005 (Altekruse, McGlynn, & Reichman, 2009; Bueno-Marí, Almeida, & Navarro, 2015). Incidence of HCC was also linked to the socioeconomic status of the people. Higher chance of occurrence was reported in individuals with low socioeconomic status. The following Figure 1 illustrate the no of studies conducted overtime to depict rise in HCC cases in the study period (Butt et al., 2012).

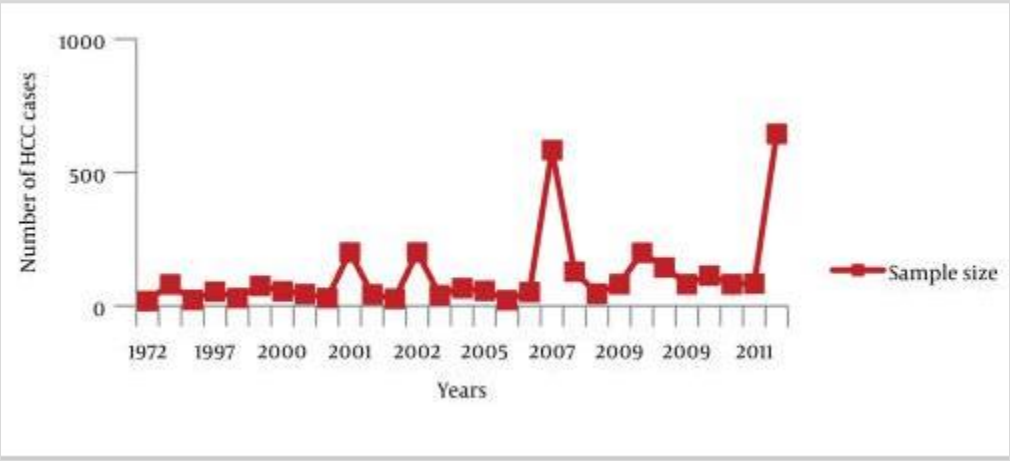


Figure 2.1: The Graph illustrate the no of studies conducted overtime and depict the rise in HCC cases.

2.1.2 Prevalence in Pakistan:

Pakistan like the rest of the world have shown a moderate to high prevalence rate for HCC where the usage of unsafe injections was considered to be the common risk factor (S. A. Ali, R. M. Donahue, H. Qureshi, & S. H. Vermund, 2009). Pakistan being a sovereign country in South Asia has a population of 180 million. This makes the country 6th most populous country (Parkash & Hamid, 2016). HCC incidence rate is predicted to rise in the

near future because of the high prevalence rate of HCV and HCB. As in some cases it is the average lag time between getting infected by the virus and development of cirrhosis that leads to the development of cancer. This trend has also been confirmed by a researcher in his study which states an increasing trend in admission of HCC patients in tertiary care hospitals (Butt et al., 2013). It is also evident from the graph below (Butt, Abbas, & Jafri, 2012).As seen globally the trend is same in Pakistan with women having lesser chance of developing Hepatocellular carcinoma in comparison to the men (Parkash & Hamid, 2016; D Max Parkin et al., 2005). It is observed globally that people usually are not seen with HCC in at least first 4 decades of life whereas on the other hand the trend is opposite in South Asia because of the high occurrence rate of viral hepatitis associated cirrhosis. Thus the age adjusted incidence rate of HCC is 3.7 for women and 5.7 for men in Pakistan (Parkash & Hamid, 2016).

Most the patient reported with HCC remained either asymptomatic or had experienced weight loss, fever, jaundice, hepatic encephalopathy and ascites(Butt et al., 2012). Most of the patients with child class B or C about 69-84 percent had cirrhosis (Abbasi, Butt, Bhutto, Gulzar, & Munir, 2010; Ali, Iqbal, & Irfan, 2010; Ansari, Memon, & Devrajani, 2009; Butt et al., 2012; Idrees et al., 2009; Khokhar, 2001). According to a study conducted it was observed that of all the patients reviewed, child class A, B and C was prevalent among 216, 147 and 37 of Patients with HCC respectively (Yusuf et al., 2007). Another study however showed different results depicting child class C to be the most prevalent with prevalence rate of 45.1% followed by child class B cirrhosis with prevalence rate of 42.3% (Butt et al., 2013). Moreover, it has been reported that approximately 82.9% of the cases were diagnosed after the patients started showing symptoms while only a small

number of patients 8.8% were diagnosed incidentally on the other hand only a ratio of 8.2% making a total of 53 individuals out of the 645 patients tested. This for sure creates an alarming situation. It was also observed that about 74.4% of the individuals with HCC were found to have at least one complication relating to cirrhosis which include portosystemic encephalopathy (34.1%), ascites (68.5%), upper gastrointestinal bleeding (40.5%), esophageal varices (53%), hypersplenism (62.2%), hepatopulmonary syndrome (7.8%), hepatorenal syndrome (22.6%) and hepatohydrothorax (9%) making the treatment difficult. About 40.2% of the patients were found to be having concomitant diabetes, 34.4% were reported to have hypertension and 2.5% of the total patients were having dyslipidemia (Butt et al., 2013). Interestingly some studies reported that patients with HBV leading to HCC were younger to the patients with HCV associated HCC (Butt et al., 2012). Patients were not under any surveillance program hence were diagnosed later in their life with an advanced stage of disease. Same was reported for NBNC chronic liver disease patients (Butt et al., 2013). Patients with HCC due to HCV were more as compared to HBV induced HCC as evident from the following Figure 2 (Butt et al., 2012).

2.2 Etiology:

HCC tends to put a significant socioeconomic burden because of its high rate of prevalence as its occurrence is equally high in developed and under developed countries. For effectively understanding HCC it is important to know pathogenesis, etiology and epidemiology of cancer.

There are multiple etiological factors involved in HCC development. HCC occurs due to typical viral infection of either Hepatitis C or B (Donald Maxwell Parkin, 2006; Tateishi et al., 2015). Hepatotropic viruses such as HCV, HDV, and HBV have found to be strongly

associated to HCC. It has been observed that HCC distribution worldwide is linked to the distribution of these viruses (Stuver & Trichopoulos, 2008). In addition to viruses other factors also contribute in HCC development. One of the major contributor is Cirrhosis causing approximately 80% to 90% of the cases (D. Y. Zhang & Friedman, 2012). Co infection of HBV/HDV and HBV/HCV can increase the HCC risk by 2 to 6 folds. Moreover the excess alcohol consumption further increases the HCC risk (Donato et al., 2002; Fattovich, Stroffolini, Zagni, & Donato, 2004). Distribution of different etiological factors for HCC are summarized in the Figure 2 below (Butt et al., 2012).

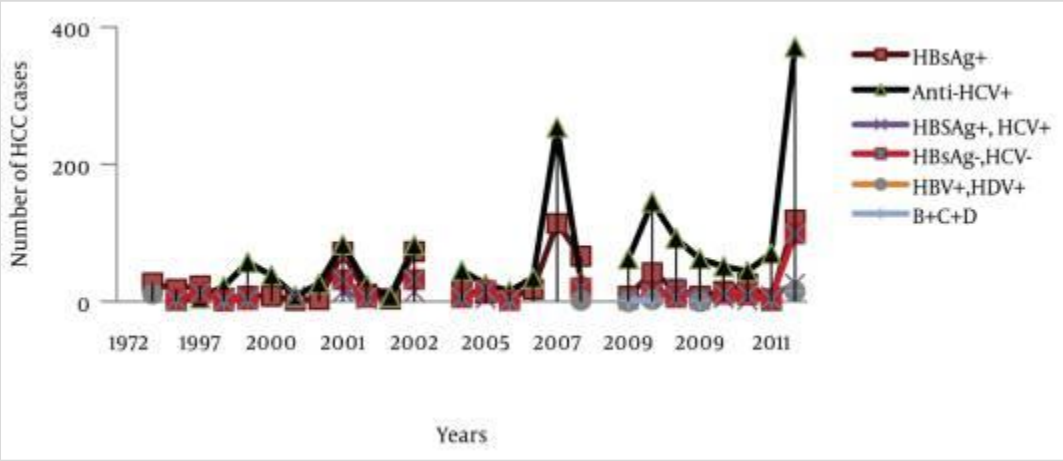


Figure 2.2: The following figure illustrate the comparison of HCC due to HBV, HCV and NBNC-HCC.

These risk factors associated with HCC are described below briefly.

2.2.1 Hepatitis B virus:

Hepatitis B virus is the most common etiological factor for liver cancer. Almost 54% of the total liver cancer is caused by HBV (Beasley, Lin, Hwang, & Chien, 1981; Donald Maxwell Parkin, 2006). It has been reported that with chronic infection of HBV the risk of

HCC development increases by 15-20 folds. The mortality rate in case of chronic HBV infection also reaches to 30% to 50% of the total cases (Y.-T. Huang et al., 2011; Lavanchy, 2004). The parts of the world endemic for HBV thus show an increased number of cases of HCC (Bosch, Ribes, Díaz, & Cléries, 2004; Ghouri et al., 2017). The pattern of transmission in such regions is perinatal and vertical in contrary to the developed countries where transmission of HBV is parental, through sexual contact or with blood infected with HBV (Bosch et al., 2004). In only US about 10% to 16% out of the total reported HCC cases are attributed to infection of HBV (El-Serag, 2012; Ghouri et al., 2017). Patients with a coinfection of HBV and HIV possess higher risk of HCC development in comparison to a person who has only HBV infection (Konopnicki et al., 2005). An individual who has been tested to be negative for surface antigen of hepatitis B but still has viral particles in blood still maintains the potential of developing cirrhosis and ultimately HCC (Chen et al., 2009; Tatematsu et al., 2009; Yotsuyanagi et al., 2000). HBV is found to have 10 genotypes. The genotype D and C have a higher chance of developing cirrhosis and ultimately HCC than any other genotype (Erhardt et al., 2005; Sonneveld et al., 2012). Genotype C however puts an individual to a greater risk of HCC development than other genotypes(Chan et al., 2008).

2.2.2 Hepatitis C virus:

Hepatitis C virus however comes at the second position in the list of most common HCC risk factors. It has been estimated that about 10% to 20% of the HCC cases worldwide are due to HCV infection (Y.-T. Huang et al., 2011; Tanaka, Tsukuma, Yamano, Oshima, & Shibata, 2004). It is the common causative agent in the developed countries (El–Serag & Rudolph, 2007; Yoshizawa, 2002). Infection of HCV rises the chances of HCC

development by 20 to 30 times. According to an estimate about 2.5% of the patients with HCV infection develop HCC (Bowen & Walker, 2005). Although there is no vaccine available but by taking certain precautionary measures and by screening the blood of the individuals for HCV infection the infection rate of HCV can be eliminated worldwide (Wedemeyer et al., 2014). People are at higher risk of developing HCC if they get co infected by HIV and HCV(Clifford et al., 2008; Soriano, Vispo, Labarga, Medrano, & Barreiro, 2010). Coinfection of people with HBV and HCV also upsurges the chances of HCC development (Kruse et al., 2014). If HCV patients are treated with interferons it decreases the risk of HCC development by 57-75% (El-Serag, 2012).

2.2.3 Alcohol consumption:

Apart from all the above reasons HCC is also strongly connected to the lifestyle of an individual. Lifestyle includes the excessive use of alcohol, diabetes type 2 and obesity (Calle, Rodriguez, Walker-Thurmond, & Thun, 2003; Donato et al., 2002; El-serag, Tran, & Everhart, 2004; Mayans et al., 1990). The trend of proportion of people with non-viral HCC is observed to be increasing globally. The third most commonly known cause for development of HCC is the excessive use of Alcohol(Bosch, Ribes, & Borràs, 1999). Alcohol aids in HCC development of patients with an infection of hepatropic viruses (Donato et al., 2002). This effect was more visible in patients who consumed about 60g of alcohol per day as heavy alcohol users were found to be having 2.3 folds higher chance of HCC development (Hutchinson, Bird, & Goldberg, 2005). Patients particularly in case of infection with HBV and heavy alcohol consumption were found to be 3 times higher risk of HCC development. This can be explained by the possible different definitions used for heavy consumption of alcohol. It is important to state here that alcohol alone has the

potential of developing HCC without the infection of any virus in the body of the individual. HCC developed by alcohol is often found to be associated to repeated inflammation, necrosis and regeneration cycles of hepatocytes accompanied by oxidative stress ultimately leading to cirrhosis (Farazi & DePinho, 2006).

2.2.4 Others:

Nonalcoholic fatty liver disease is a commonly occurring reason of developing cirrhosis which ultimately leads to HCC development. With the rise in the incidence rate of metabolic syndrome, obesity and diabetes the number of people with HCC are also on rise. A study from Japan stated that Nonalcoholic steatohepatitis (NASH) had similar impact on development of HCC as infection with HCV(Yatsuji et al., 2009). The risk of HCC development by NASH was found to be 11.7% when compared to the risk in chronic HCV infection which was found to be 30.4% similar to another international study (Bhala et al., 2011). An interesting fact found was that in patients who had NASH no increase in chances of developing HCC was observed but were not having cirrhosis (Dam-Larsen et al., 2009).

2.3 Diagnosis and Treatment:

Like every cancer it is always beneficial if HCC is detected in its early stage as it increases the chances of survival of the patient (van Meer et al., 2015; B.-H. Zhang, Yang, & Tang, 2004). The reason is that the patients who ae diagnosed at initial stages have multiple options for treatment. But many people are not so lucky as the rate of people getting effective treatment and that too on the right time is only 30% to 40% of the total (Llovet et al., 2008). The current techniques available give far from satisfactory results. These include

current serum biomarkers, vitamin k deficiency induced proteins and imaging techniques (computed tomography, ultrasonography and magnetic resonance imaging).

Many countries have started surveillance programs for screening of HCC as this is a cost-effective way in comparison to HCC treatment. For treatment of HCC surgeons usually make either liver transplantation or resection of cancerous tissue as their primary choice. But due to the higher rate of HCC incidence in patients after cirrhosis, the resection of the tumor becomes a very challenging task. Due to this liver transplantation is left as the only choice for treatment. Liver transplantation although seems to be a better option but it is not feasible due to lesser availability of donor organs.

2.3.1 Tissue biopsy:

Personalized therapy is considered to be the future of cancer therapy. Now a days for the study of cancer, tumor tissue is obtained from the patient and is analyzed for tumor linked genetic alterations as for development of cancer certain genetic as well as epigenetic anomalies are responsible (Coppedè et al., 2014; Herman & Baylin, 2003; Ozen et al., 2013; Sharma et al., 2010; Yoo & Jones, 2006). Although this method is thought to be a gold standard for cancer diagnosis but the information provided by the tumor biopsy is often limited and is unable to reflect disease heterogeneity (Awad et al., 2013; Bardelli & Pantel, 2017; Siravegna et al., 2015).

There are different biomarkers that are in use for detection of tumor such as des-γ-carboxy prothrombin, Alpha-fetoprotein (AFP) and AFP lectin fraction (AFP-L3). The drawback of using all these makers is that they are not highly specific as well as sensitive and thus are prone to giving false positive results (Sterling et al., 2009; Tateishi et al., 2008; Yoon,

Han, & Kim, 2009). In addition to this all these tumor biomarkers were discovered by working on the tissue samples obtained from HCC patients through biopsy. It is not possible to obtain a tissue sample every time for work (Kelley et al., 2015; Yin et al., 2016) and due to this the sample size remains low which causes the results to be less reliable. Due to the invasive nature as well as poor reliability of results these conventional methods have to be replaced by more efficient methodologies. One such method is liquid biopsy.

2.3.2 Liquid biopsy:

Liquid biopsies are considered to be a game changer as they provide a minimally invasive and sensitive approach alternative to tissue biopsy (Alix-Panabières & Pantel, 2016; Bardelli & Pantel, 2017; Diaz Jr & Bardelli, 2014).The results obtained through liquid biopsy are quite promising and hence great effort is done to design specific biomarkers for early cancer detection as well as for increasing the sensitivity and efficiency of treatment. Other advantages of liquid biopsy include for efficient monitoring of the response of the administered treatment and the resistance against the therapy requires repeated sampling which becomes a limitation in tumor biopsy due to its invasive nature but is no issue in liquid biopsy. Moreover liquid biopsies provides a comprehensive and accurate picture of the tumor microenvironment thereby enabling us for (1) early stage cancer detection (2) discovering of new targets to be used in personalized treatment (3) individual patient's prognosis : classification of cancer into stage and potential for metastasis (4) estimation for reoccurrence of disease (5) pre-treatment classification of cancer for carrying out personalized therapy (6) real time monitoring of the response to treatment.

Many mediums can be explored for their use in liquid biopsy but blood serves as a rich medium through which not only we can detect cancers but also can classify them into

subtypes (Antonarakis et al., 2014; Chu et al., 2016; Gorges et al., 2015; Gorges et al., 2016; Guttery et al., 2015; Maheswaran et al., 2008; Miyamoto et al., 2012; Schiavon et al., 2015). Not only blood-based biomarkers have proved to be promising biomarkers to be used for prognosis, diagnosis and for treatment purposes but also obtaining blood sample is way easier than obtaining a biopsy sample. This will not only increase the sample size but also the results obtained would be more reliable as they are able to correctly describe the tumor dynamics (Alix-Panabières & Pantel, 2013; Crowley, Di Nicolantonio, Loupakis, & Bardelli, 2013; Schwarzenbach, Hoon, & Pantel, 2011; van de Stolpe, Pantel, Sleijfer, Terstappen, & Den Toonder, 2011).

Different biomarkers have been explored for finding a more effective way of detecting as well as treating HCC. Current biomarkers used in liquid biopsy include Circulating tumor DNA, extracellular vesicles, circulating tumor cells and Tumor educated platelets (TEP).

2.3.2.1 Circulating tumor cells (CTCs):

Circulating tumor cells can be considered as tumor seeds that are continuously shed by the tumor into the blood stream. These circulating cancer cells then further help in establishing metastatic niche away from its site of origination (Yin et al., 2016). They were detected for the first time in 1869 by Ashworth (Ashworth, 1869). The issue is that these cells tend to increase in blood with the increase in stage of cancer. So in a person with early stage cancer the amount of CTC in blood would be low moreover major portion of the cells do not survive and only 0.01% of CTC produce metastasis (Okajima et al., 2017) leaving very less chances for CTC detection in a patient with early stage carcinoma. In addition to this CTC are heterogeneous in both their genotype and phenotype (Fidler, 1970; Luzzi et al.,

1998; O'Flaherty et al., 2012). Therefore, the use of CTC in liquid biopsy is hampered by the inability of a researcher to detect them precisely.

2.3.2.2 Cell free nucleic acids (CfDNA/RNA):

When using cell free nucleic acids as a biomarker in liquid biopsy it is observed that the cellular turnover only increases after the tumor increase in volume. This further increases the quantity of apoptotic and necrotic cells. According to an observation the cfDNA fragments usually have a size between 180 and 200 bp which suggests that the majority of cfDNA is produced by apoptosis. Interestingly, in some tumor types shorter cfDNA fragments have been reported (e.g., hepatocellular carcinomas). On the other hand cfDNA fragments having a size of thousands of base pairs are usually considered as the necrosis outcome (Jiang & Lo, 2016). These cell free nucleic acids thus are difficult to detect without a large cellular turnover which means the cancer would have by then reached a higher class.

2.3.2.3 Tumor educated Platelets:

A new promising approach that can be used in liquid biopsy for earlier detection of liver cancer is the use of nucleic acid content in platelets of the patient.

2.4 Platelets:

Platelets are highly available for the diagnostic purposes due to their abundance in blood which ranges from 200 to 500 million per ml of blood. Platelets (anucleate cells of 2 to 4mM) originate from large progenitor cells often known as megakaryocytes present in bone marrow and are abundantly found in blood (1.5–4.0 $X10^9$/L) (Gazzaniga & Ottini, 2001). Hewson is considered as to be probably the first person to detect the presence of undefined

small particles in blood in 1780 (Buchanan, 1836). In 1841 George Gulliver and in 1842 William Addison drew platelets and platelet and fibrin clot respectively (Osler & Schaefer, 1873) and in 1842 Alfred Donne named them "globulin du chyle" which means small globules originated from plasma (Hammarsten, 1875). In 1850 Beale described them as particles of "germinal matter" whereas in 1860 they were named as "small corpuscles" by Zimmermann (Pekelharing, 1892). Lionel Beale was the first person to be able to successfully publish his drawings of platelets. None of these scientist succeeded in providing correct and detailed description until Max Schultze who in 1865 was able to correctly describe these platelets (Gazzaniga & Ottini, 2001).

He described them as clumps of size of approx. 80u, made up of irregularly shaped small globules of 1-2 in diameter having radial extensions with no spontaneous motility. He also successfully related them to blood clotting. There were no experiments later conducted by the scientist to ensure their nature and hence he also considered them as degenerated leukocytes. Later these vesicles of Schultze were named differently as in 1873 Ranvier considered them as clots of fibrin and even some scientists such as Osier and Schafer in 1873 considered them to be some specific type of bacteria (Van Leeuwenhoek). George Hayem a renowned hematologist of his time however clearly described these vesicles in 1878 and 1879 (Hewson, 1771). He described these vesicles as pale colored delicate erythrocytes which rapidly change their shape and their size range from 1.5 to 4.5 in diameter and have the ability of forming aggregates. These observations by Hayem were however rejected by Riess in 1879 who said the particles originated from leukocytes. This statement was further rejected by Neumann, according to him these were just the artifacts

which originated from the red blood cells due to the incorrect technique of blood withdrawal used at that time (Donne, 1842).

Giulio Bizzozzero in 1882 for the first time succeeded in defining these corpuscles (G. Bizzozero, 1883) to be devoid of nucleus, unrelated to red blood cells and leukocytes and considered them to be the third morphological element of the blood. He named them as *piastrine* which means small plates (Schultze, 1865). Bizzozezzero had a great role in understanding the part played by platelets in homeostasis and thrombosis mechanism. He rectified the long-known concept of thrombus containing fibrin and leukocytes only developed by Virchow in 1856, Weiger in 1877 and Cohnheim in 1882. James Wright became the first person to use the term platelets for the first time in his publication in 1910. The term platelets is universally accepted and used (Gazzaniga & Ottini, 2001).

2.4.1 Platelet production:

Platelets were found to originate from megakaryocytes. Megakaryocytes protrude long branches of 2-4um, called as proplatelets. These proplatelets constitute of swellings of approximately the size of the platelet in tandem arrays. These branches are connected via cytoplasmic bridges (G. Bizzozero, 1881). Platelets are thought to be released from these proplatelets. Actin is involved in formation of platelets from proplatelets but still its complete role in the process needs to be exploited (J. Bizzozero, 1882). Platelets contain several organelles including mitochondria and other granules. These are transferred via microtubules in the proplatelet shaft at a rate of 0.1-0.2um/min (Gazzaniga & Ottini, 2001). This transfer is usually bidirectional and is involved in mixing of all the organelles in the proplatelet prior to its transfer to the ends of proplatelet from where platelets are thought to be released into blood circulation. This process takes 4 to 10hrs for completion. The

complete mechanism of platelet production is yet not known as some questions remain unanswered. These questions include (1) what are the factors which induces the release of platelets? (2) The mechanism by which this process is carried out? (3) How does the platelet phenotype changes in diseased conditions and what induces this change and by which mechanism?

2.4.2 Functions of platelets:

Platelets are involved in various potent functions of the body including the hemostasis and arterial thrombosis. In addition to these functions platelets are seen assisting in several physiological as well as pathophysiological processes i.e. immunity, inflammation, vessel remodeling and angiogenesis as a whole.

2.5 Association of platelets with cancer:

It has been proved through multiple studies that platelets play a critical role in cancer progression via helping in metastasis (Boucharaba et al., 2004) (Belloc et al., 1995). Platelets are known to play role in tumor metastasis by assisting in intravasation, extravasation, protection from immune system and ultimately aid in establishing a tumor at secondary site (Goubran et al., 2014; Skog et al., 2008).

Platelets can be linked to both tumor metastasis and the tumor biology (Kuznetsov et al., 2012; McAllister & Weinberg, 2014) as contents of platelets are seen to be affected by the tumor cells especially their RNA and Protein (Best et al., 2015; Calverley et al., 2010; Nilsson et al., 2011). Tumor release RNA in blood through different microvesicle dependent and independent pathways. These released RNA are then ingested by the circulating platelets hence known as tumor educated platelets (Best et al., 2015; Nilsson et

al., 2011). As a result these tumor educated platelets (TEP) help in promoting survival of cancer cell and its migration to distant sites (Hanahan & Weinberg, 2011). Indirect interaction of platelets with tumor involve various signaling molecules whereas the direct interaction occur via certain receptors especially P-selectin "a platelet activation receptor" (Borsig, 2008; Egan et al., 2011; Steinert, Tang, Grossi, Umbarger, & Honn, 1993; Zucchella et al., 1989). After getting activated the platelets release various angiogenic and growth factors like PDGF, EGF, BFGF, ANGPT1, IGF1, VEGF-A,HGF, VEGF-C, TGFβ (Menter et al., 2014; Sierko & Wojtukiewicz, 2004). This activation of platelets and release of different molecules at a metastatic niche help in creating a pro-tumor micro environment (McAllister & Weinberg, 2014).

Moreover platelets were seen protecting the tumor cells from immune surveillance by the transfer of proteins of MHC class I to tumor cell (Placke et al., 2012). Apart of protection by transferring molecules, platelets protect tumor cells physically by surrounding them to form an aggregate of tumor cell, fibrin and the platelets. This cloaking of tumor cells forms a physical shield around them and protects it from getting detected which ultimately results in increased tumor cell survival (Borsig et al., 2001; Labelle, Begum, & Hynes, 2011; Palumbo et al., 2007; Palumbo et al., 2005). Platelets with all these protective properties enhances tumor cell survival in blood circulation and ultimately supports metastasis (Buergy, Wenz, Groden, & Brockmann, 2012; Gasic, Gasic, & Stewart, 1968).

Platelets do not have a nucleus but are equipped with RNA of different types which include mRNA, pre-mRNA and miRNA. Major portion of these RNA transcripts are transferred from megakaryocytes during their production. Tumor release several biomolecules which are ingested by the platelets and in result of it they are considered to be educated by the

tumor (Best et al., 2015; Brown & McIntyre, 2011; Nilsson et al., 2011; Nilsson et al., 2016). Several external stimuli received by the platelets e.g. lipopolysaccharide-mediated platelet activation and activation through surface receptors causes specific splicing of mRNA present within educated platelets (Brown & McIntyre, 2011). Certain splice events that are queue specific may occur in TEP due to the signals released by tumor and its microenvironment which include stromal and the immune cells (McAllister & Weinberg, 2014). Moreover, platelets have the ability of ingesting the free circulating RNA present in blood or while interacting with a cell. These RNA biomarkers present in platelets are also seen to alter in presence of cancer. After their first discovery by Bizzozero in 1881 they have been widely studied in both thrombosis and hemostasis.

2.6 Use of platelets for cancer detection:

There exist several studies that prove the platelets potential as a diagnostic tool for cancer detection. Platelet count has been seen harboring information important from clinical point of view (Bottsford-Miller et al., 2015; Cho et al., 2012; Kemona, 2013; Maria Mantur et al., 2008; Ryningen et al., 2006; Stone et al., 2012). In addition of the platelet count, platelet protein markers e.g. P-selectin (Dymicka-Piekarska, Matowicka-Karna, Osada, Kemona, & Butkiewicz, 2006; Kamińska et al., 2014; Kemona, 2013; M Mantur, Kemona, Kozłowski, & Kemona-Chetnik, 2003; M Mantur, Kemona, Pietruczuk, & Wasiluk, 2002; Osada, Rusak, Kamocki, Dabrowska, & Kedra, 2010; Peterson et al., 2012) and the size of platelets (Kemona, 2013; R. Wang et al., 2015) have been used for diagnosis and prognosis of cancer. Platelet hyper-reactivity is achieved by interaction of cancer cell and platelets through various pathways (Cooke et al., 2013; Meikle et al., 2017). Number of young reticulated platelets is seen to be on rise in cancer patients (Angénieux et al., 2016) but this

ratio is observed to change after treatment (Balduini et al., 1999; Dymicka-Piekarska et al., 2006; Ryningen et al., 2006).

Platelets due to their ingesting ability are known to take up nucleotides and proteins during circulation. Platelets ability to uptake extracellular vesicles was described by Nilsson et al (Nilsson et al., 2011). The mechanism proposed indicated that platelets were able to sequester mRNA harboring EVs derived from tumor cells. This was proved after the detection of EGFRvIII (a mutant of EGFR found in 30% of glioblastoma tumors) via RT-PCR of platelets obtained from cancer patients with 80% sensitivity and 96% specificity. Furthermore the microarray analysis of the mRNA repertoire was able to distinguish between the healthy and the diseased (Nilsson et al., 2011). Out of the 30 genes with differential expression 17 were present in TEP with 4 of them to be significantly differentially expressed i.e. IL1R2, TPCN1, FKBP5 and WEDC1. This ingestion of tumor derived RNA by the platelets was also confirmed in patient with NSCLC in 2015. Deep amplicon sequencing and RT-PCR were able to detect EGFR, KRAS and translocated EML4-ALK transcripts with point mutations that were tumor specific (Best et al., 2015; Nilsson et al., 2016). RNA profiles of platelets helped in the formation of surrogate gene panels for determining the molecular status of the tumor tissue in situ as shallow sequencing of platelets was not enough for revealing specific mutations.

Platelets can also play a critical part in detection of the rearrangements in genes e.g. ALK gene. The rearrangement in this gene increases its sensitivity making it more sensitive to ALK TKIs crizotinib (Solomon et al., 2014). Rearrangement in Platelets of NSCLC patients in (EML4)-ALK was observed by Nilsson (Nilsson et al., 2016). He screened for all the rearrangements in cfRNA and exosomal RNA in both the plasma and platelets of

the 77 patients out of which 38 patients were detected to have these rearrangements with 65% sensitivity and 100% specificity in platelets and with 21% sensitivity and 100% specificity in RNA extracted from plasma of patients. The low sensitivity can be explained by either quick degradation of cfRNA or the decreased number of exosomes which had this rearrangement in it.

2.6.1 Non-coding RNA of platelets in liquid biopsy:

Platelets potential to be used as liquid biopsy was shown by thromboSeq technique. In addition to mRNA platelets are also known to harbor small and non-coding RNA. After the analysis of these non-coding RNA it was revealed that 16 were upregulated out of 20. All these 20 non-coding genes were found to have a profile that was tumor specific. One of the down regulated RNA was (MALAT1)(Best et al., 2015). This is known to act as regulator for transcription for various metastasis related genes hence increase in its expression enhances proliferation and metastasis of tumor genes (S. Chen et al., 2017) (D. Chen et al., 2017; J.-k. Huang et al., 2016). Another non-coding RNA downregulated is Growth arrest-specific transcript 5 (GAS5) which after getting downregulated act as pro cancerous in various tumors (Bian, Shi, Shao, Li, & Song, 2017; Luo et al., 2017). Tumor cell migration as well as proliferation is regulated by both SNHG8 (small nucleolar RNA host gene) and SNHG5 (T. Huang et al., 2016; L Zhao et al., 2016; Lianmei Zhao et al., 2016). It is frequently observed in several hematological cancers that lymphocytic leukemia 1 and 2 are deleted and cancer susceptibility candidate 15 plays role in formation of neuroblastoma (Kasar et al., 2014; Morenos et al., 2014; Russell et al., 2015). These non-coding RNA should be further exploited for their role in cancer.

2.6.2 Micro RNA of platelets in liquid biopsy:

Micro RNA are of special interest from all the noncoding RNA. It was described in 2012 by Ple et al. that approximately there are a total of 532 micro RNA present in platelets (Plé et al., 2012). Researchers have proposed importance of these micro RNA in platelet function as well as in their biogenesis (Seema Dangwal & Thum, 2012; S Dangwal & Thum, 2013; Edelstein & Bray, 2011; Edelstein et al., 2013). 9 micro RNA were also found to have differential expression in patients with myocardial infarction in comparison to healthy individuals(Gidlöf et al., 2013). This indicate possible use of micro RNA as a diagnostic tool. Although micro RNA are found to be involved in cancer yet most of the studies conducted now a days are on exploring their expression in exosomes and tissue. Further research should be conducted to explore the micro RNA expression in platelets educated by tumor.

2.6.3 mRNA of platelets in liquid biopsy:

As far as the most abundant type of RNA in platelets is concerned the tumor may release the mRNA in the blood vessel both in micro vesicle dependent and independent mechanism. After this engulfing, platelets go through certain changes and are known to get educated by the tumor (Best et al., 2015; Nilsson et al., 2011). These changes in combination with the ability of platelets of direct mRNA ingestion and to undergo precise splice events as a response to external stimuli would result in highly dynamic mRNA repertoire of TEP. This mRNA repertoire can be used for cancer diagnosis. For the isolation and analysis of this mRNA of TEP a highly sensitive method was devised. This method was able to distinguish between the mRNA profiles of the diseased from the healthy individuals (Best et al., 2015; Calverley et al., 2010; Nilsson et al., 2011). Platelets thus

can be utilized for harvesting the information that can tell the current disease status of the patient

mRNA profile of platelets was compared by Calverley et al. from both the healthy and treatment naïve patients with metastatic lung cancer (Calverley et al., 2010). It was revealed by unsupervised hierarchical clustering that 200 altered RNA are present from which 3 were found to be decreased in platelets of lung cancer patients. Differential splicing of SIRT2 in patients with lung cancer as compared to healthy individuals was also shown by them. SIRT2 is basically a human homolog of yeast protein Sir2. It is produced in response to cellular stress and is found to be involved in suppression of rDNA recombination and epigenetic gene silencing. Although it's exact function in humans in not known but it is known to regulate acetylation of FoxO1, p53 and various other tumor suppressor genes as the above gene has established role in epigenetic silencing which concludes that growth and metastasis can be initiated by platelets by the release of epigenetic silencers(Calverley et al., 2010).

The alteration in the RNA profile of platelets due to interaction with tumor also enabled researchers to distinguish patients with cancer from healthy individuals and that too with an accuracy of 96% (Best et al., 2015). These extracted mRNA after sequencing allowed them to scan for the biomarkers that can be generally used for cancer detection. These biomarkers not only distinguished patients with cancer from healthy individuals but also, they were able to characterize the tumor into its type as well as subclasses. Accuracy rate for this characterization was found to be 71%. s

It was reported in a recent study that out of 283 samples of platelets obtained from the blood from both healthy and the cancer patients i.e. lung cancer, glioblastoma, pancreatic

cancer, breast cancer, colorectal cancer and hepatobiliary cancer, 1453 RNA were found to have an increased expression in TEP whereas 793 RNA showed decrease in expression when compared to RNA obtained from healthy individuals (Best et al., 2015). These figures represent a strong correlation between the mRNA profile of platelets and tumor. Moreover, a predictive algorithm was developed by the researchers which allowed to cancer detection (96%), tumor type (71%) and subtype (85-95%) accuracy. This algorithm detected cancer in all the patients with cancer at an early stage (Best et al., 2015). The results were very promising but yet require a thorough evaluation by performing these algorithms on a larger sample number and different other tumor types as the algorithm was although not able to distinguish between localized and metastatic tumor but a larger set of samples of same cancer type might be able to do so.

mRNA profile of TEP was found to be specific to the tumor type so it is hypothesized that such a multiclass algorithm can be developed which will not only be able to detect the primary tumor site but also could separate tumors on basis of their mutational status (Best et al., 2015). With the difference in RNA profile of primary tumors some resemblances were also observed. These resemblances can be explained by the fact that those tumors may have similar driver mutations which can be detected in platelet via deep sequencing. For example, KRAS is a driver mutation observed in various types of cancer. This and many other mutations like this leave a specific signature within the platelets. This signature would make selection of patients for different targeted therapies easier.

RNA panel of TEP was subjected to DAVID and CAGE gene ontology algorithms to determine their potential in diagnosis. A decrease in expression was observed of RNA involved in metabolism and splicing of RNA. Moreover, a correlation was observed

between the platelet activation, transport of platelets and vesicles, activation of cytoskeleton and signaling via ATP. These programs indicate the hyper reactive state of TEP which resembles to the hyper reactivity indicated in platelets of cancer patients after their functional analysis. This hyperactivity appears to vary in patients with cancer in comparison to the patients with non-cancerous inflammatory disease.

22 genes were found to have difference in expression in platelets of patients with non-cancerous inflammatory diseases (Gnatenko et al., 2010; Healy et al., 2006; Lood et al., 2010; Raghavachari et al., 2007). These genes were found to be randomly expressed in TEP which indicate that the RNA profile of platelets from non-cancerous inflammatory diseases is different from patients with cancer (Best et al., 2015).

Further research on RNA profile of TEP as well as on patients with non-cancerous inflammatory disease should be conducted which after comparison to the healthy individuals would result in better knowledge about the role played by platelets in cancer and other diseases and their efficient use as a biomarker in early diagnosis of the disease. Despite the need of further research for validating these results yet these results highlight the ability of platelets to be used as liquid biopsy. Further issues linked to liquid biopsy can be fixed with the development of highly sensitive as well as specific techniques (Forshew et al., 2012; Kinde, Wu, Papadopoulos, Kinzler, & Vogelstein, 2011; Newman et al., 2014).

Hence we can safely say that the findings of (Best et al., 2015) are quite promising. They have a potential to be used in future cancer therapies. Patients in future will receive therapies based on mRNA profile of the tumor educated platelets as part of clinical trials. Over the last decade many novel drugs for chemotherapy i.e. small inhibitors, antibodies have been developed for

targeting specific receptors present on cancer cells (Joosse & Pantel, 2015). Moreover, these drugs ae developed for targeting metastatic tumor cells. Currently there is a need to develop methodologies that can better analyze the primary tumor. It is observed that the metastatic cells harbor certain alterations in genome when compared to the primary tumor because they get metastasized years after their diagnosis (Joosse & Pantel, 2015). Therefore, the analysis of this metastasized cell will aid in revealing significant information. This information will be helpful in generating a systematic cancer therapy. It has been reported that certain metastatic sites have different alteration in the genome hence analyzing the tumor cells obtained by one or two biopsies will not be true representative of the alterations that occur in a genome of tumor cell. In such a case analyzing the tumor educated platelets for their mRNA profile can be considered as quite promising as it would be able to provide the information in real time about the current status of the metastatic tumor cells. Future research is required for determining the role of TEP in assisting in stratification of patients for the appropriate therapy (Joosse & Pantel, 2015).

Although before starting the clinical trials, the factors that can change the mRNA profile should be assessed properly. For example, it is recommended by many authors that blood isolated from the patient can be isolated within 48hr but research confirms that this time is enough for many cell types to alter their mRNA profile. It should also be kept in mind that it is not only cancer that changes the mRNA profile of platelets but many inflammatory diseases whether chronic or transient and cardiovascular events are enough for causing the alterations (Joosse & Pantel, 2015).

Considering the potential of TEP in determining the location of primary tumor would serve as a great breakthrough in practicing liquid biopsies for diagnosis of cancer after validation. Validation of these assays of liquid biopsy is the task of European consortium_CANCER-ID. It contains more > 30 institutions that belong to both industry as well as academia.

All the selected biomarkers in our study i.e. TGF-β, NF-κβ, VEGF, AKT and PI3K are all well known to have a pro-tumor effect. Moreover, these were observed to be involved in interaction of platelets with the tumor. The following figure is adapted from (Fuentes, Rojas, & Palomo, 2016; Ghafoory et al., 2018; Wiesner et al., 2010; Woulfe et al., 2004) as described below:

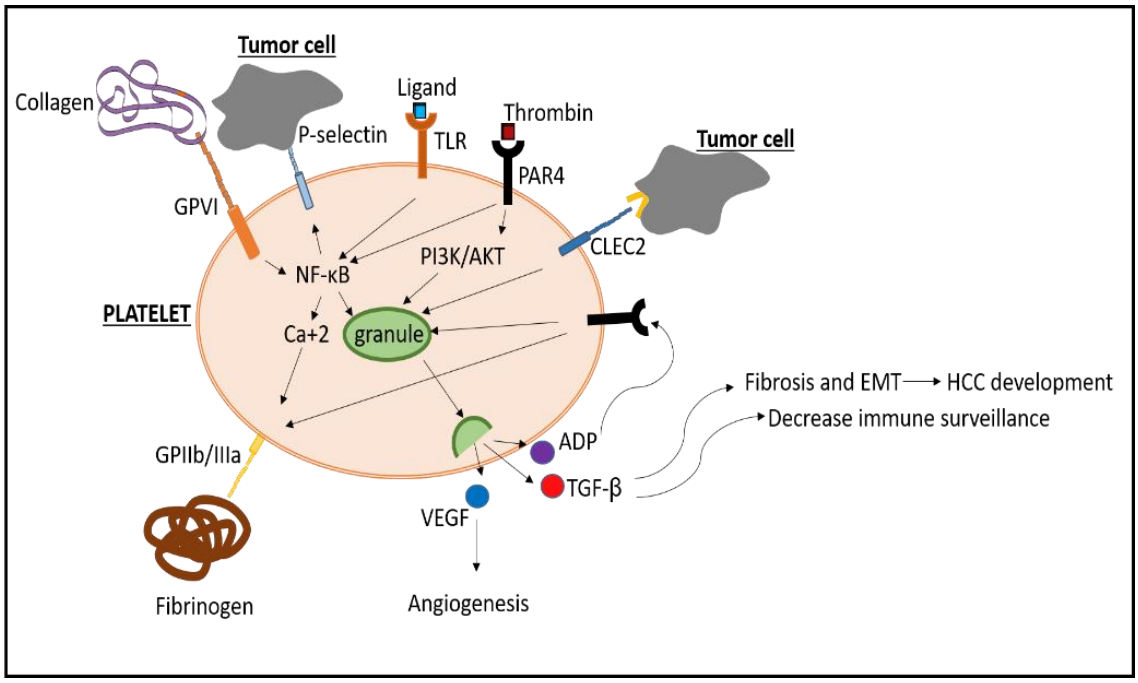


Figure 2.3: The figure illustrates the interaction of platelets with tumor cells

Chapter 3

MATERIALS AND METHODS

3.1 Primer sequences:

The sequences of the primers used for optimization of selected biomarkers are shown in the table below:

Genes	Primer Sequences	Product size
FP-VEGF	5'CCCACTGAGGAGTCCAACAT3'	173bp
RP-VEGF	5'AAATGCTTTCTCCGCTCTGA3'	
TGFβ1-F	5'TATCGACATGGAGCTGGTGA3'	240bp
TGFβ1-R	5'TGGGTTTCCACCATTAGCAC3'	
NFκβ-F	5'AGAGGCGTGTATAAGGGGCTA3'	155bp
NFκβ-R	5'TGAGGTCCATCTCCTTGGTC3'	
PI3KR1-F	5'CTGCCTCCTAAACCACCAAA3'	153bp
PI3KR1-R	5'TACCAAAAAGGTCCCGTCTG3'	
AKT-F	5'ACAAGGACGGGCACATTAAG3'	188bp
AKT-R	5'ACCGCACATCATCTCGTACA3'	

Table 3.1: Selected primers for all the genes with their sequences and expected amplicon sizes

3.2 Collection of blood samples:

IRB approval for the study was obtained from the parent department "Atta-ur-Rehman School of Applied Biosciences (ASAB) NUST. For obtaining blood samples Rawalpindi Medical University (RMU) was contacted. After getting an IRB approval from their ethical committee Board, samples were collected from Center of Liver Disease (CLD) at Holy Family Hospital Rawalpindi. Patients were asked for their consents before obtaining the sample. 6ml of blood was drawn slowly with care. The blood was then shifted immediately to the purple capped EDTA vacutainer tubes to prevent clotting of the blood sample. Tubes were labelled properly with patient's name and ID. These collected samples were then transported back on ice to ASAB within two hours of sample collection for further processing. Blood samples obtained from ASAB were used as healthy controls. All these samples were tested for ELISA and LFT at the diagnostic lab of ASAB before utilization. The patients included in this study were confirmed HCC patients. All the patients were subjected to the exclusion and inclusion criteria as described below:

Table 3.2: Table summarizes exclusion and inclusion criteria used.

Exclusion Criteria	Inclusion Criteria
• Patients of age below 18	• Selected patients would be in age range of 18 and above
• Patients suffering from any bacterial or fungal infectious diseases i.e. Tuberculosis would be excluded	• Written and informed consents will be obtained from patients

• Patients who are not willing to be the part of research	• Patients should have confirmed case of liver cancer: BCLC staging 0 to D
• Patients on medication especially aspirin or any steroids will be excluded	• Patients of any etiology leading to liver Cancer i.e. HCC
• Patients on blood transfusions because of low Hb levels	• Patients with known LFT.

3.3 Separation of Platelets from blood:

Density gradient centrifugation with the correct speed can lead to fractionation of blood. These fractionated blood cells can be separated to get the pure platelets population. 6ml of the blood present in EDTA tubes was poured into 15ml falcon tubes. The falcon tubes were then subjected to centrifugation at 120Xg for 20 min at 4°C (Eppendorf centrifuge 5810R). The acceleration and deceleration speed were kept minimum to avoid the activation of platelets. This centrifugation speed was enough to separate the red blood cells and a buffy coat layer containing the lymphocytes from the rest of blood cells. The upper plasma was collected without disturbing both the layers and was transferred to another 15ml falcon. This was later given another spin of 360Xg with zero acceleration and deceleration to separate out the platelets from rest of cells. A white pallet of platelets can be seen at the bottom of the tube after the spin ends. Plasma was aspirated to leave only the pallet in the tube. 100ul of ACD buffer was used to resuspend the pallet. ACD buffer due to its peculiar properties is helpful in retaining the platelets in their inactive state.

3.4 Checking the purity of isolated platelets:

The resuspended platelets were then visualized under microscope to see if they had any contamination of red or white blood cells in them. This visualization also helped in getting the idea of the number of cells that were in their inactive and activated state. Getting 3 or 4 cells on the background of millions of platelets was considered to be pure enough for further processing.

3.4.1 Flow cytometry-CD151 staining:

For investigating the presence and purity of platelets, obtained through centrifugation of the sample, flow cytometry was carried out by the use of BD Flow Cytometer (BD). Flow cytometry helped in Qualitative and Quantitative analyses of platelets after staining them with CD151 antibody. After the centrifugation platelets number of more than 5X 10^5 platelets were obtained and were re-suspended in ACD buffer to minimize activation. Washing was performed with 0.1% PBS-BSA solution by adding 200ul of the solution in re-suspended platelets and centrifuging them at 360g for 20min. Pallet of platelets obtained after completion of the centrifugation step was re-suspended in 50-100ul of PBS-BSA. 5ul of the antibody CD151 and 1ul of the antibody IgG were added in separate eppendorf's and were incubated at 4℃ in dark for 1 hr. After incubation platelets were washed with 0.1% PBS-BSA in dark by centrifuging them at 360g for 20min. Add 1.5ml of formaldehyde solution to the platelets pallet and let it rest for 10min followed by immediate analysis by Cellquest BD FACS software (BD Biosciences).

3.5 RNA extraction and quantification:

MagMax mirVana Total RNA isolation kit was used for the isolation of Rna from platelets. The resuspended platelets were transferred to a 2ml eppendorf tube. A solution was prepared by mixing 100ul of isopropanol with 1ul of mercaptoethanol and 99ul of lysis buffer. This was added to the resuspended platelets and were mixed by vigorous pipetting for 4 or 5 times. Later it was allowed to rest for 5min at RT. After the incubation the samples were shaken for 3min. 20ul of mixture of binding beads was added to the samples in eppendorf. The eppendorfs later were put on shaker for 5min. After the uniform mixing of the samples with the beads the eppendorfs were centrifuged for 2min at 13300g to separate the beads from the sample mixture. The sample solution was aspirated out carefully without troubling the beads resting at the bottom of the eppendorf tube. Add 150ul of wash solution 1 to each of the eppendorf tube. After shaking the tube for 1min, it was centrifuged at 13300Xg for 2min to settle the beads down at the bottom of the tube. The solution was carefully aspirated out without touching the beads at the bottom. Later washing of the beads was performed by adding 150ul of Wash Solution 2. Tubes were shaken for 1min and centrifuged for 2min at 13400Xg. After completion of the washing step the wash solution was carefully aspirated out. The beads were allowed to dry for 2min. It should be noted here that over drying of the beads should be avoided so do not exceed the time limit of 2min. 50ul of TURBO DNase solution was added to each sample tube and was given a shake for 15min. After shaking 50ul of rebinding buffer and 100ul of isopropanol was added to the tubes. These two solutions should not be mixed prior to addition. The tubes were again shaken for 3min and later centrifuged at 13300Xg for 2min. Supernatant was discarded and 150ul of wash buffer solution 2 was poured in the tubes. After 1 min of shaking the tubes were centrifuged at 13300Xg for 2min. Supernatant

obtained was again discarded and washing with Wash buffer solution 2 was repeated. After completion of washing step, the beads were dried for 2min. 50 to 100ul of preheated Elution buffer was added to the tubes depending upon the desired final concentration of RNA. The eppendorf tubes were again shaken for 5min to completely elute the RNA from the beads into the elution buffer. Later the beads were separated from the elution buffer via centrifugation at 13300Xg for 4min. Elution buffer containing the extracted RNA was carefully aspirated and placed at -20℃.

3.6 cDNA preparation:

Extracted RNA was not checked on Nano drop as reading of Nano drop is not reliable for very low quantities of RNA sample. So, all the RNA sample was used for preparing cDNA in two separate reactions using the reverse transcription process with the help of oligo (dT) primers. Following protocol was followed for cDNA synthesis.

RNA_____ 30ul/20ul
Nuclease free water_____11ul/22ul
10mM DNTPs_____2ul
5Xreaction buffer_____4ul
Primer oligo dT_____1ul
Revertaid_____ 1ul
Reverse transcriptase_____1ul
Final volume_____50ul

The PCR used for preparation of cDNA was THERMOCYCLER GeneAmp®PCR system9700. Profile used was 25℃ for 5min, 42℃ for 60min and at 70℃ for 7min. After reaction is complete the PCR tubes containing the cDNA are stored at -20℃.

3.7 Optimization of selected genes on conventional PCR

3.7.1 Optimization of GAPDH:

Following set up for PCR reaction was used:

Taq buffer_____2ul

2.5mM MgCl2_____2.8ul

2mM dNTPs_____2ul

Forward primer_____2ul (20pmole)

Reverse primer_____2ul (20pmole)

cDNA_____ 5ul (approx 0.5ug)

Nuclease free water_____3.7ul

Taq Polymerase_____0.5ul (2.5unit)

Final reaction volume_____20ul

PCR machine used for carrying out the experiment was 9700 Applied Biosystem thermocycler. PCR profile used was preheating the reaction mixture at 95℃ for 5min, later 35 cycles were performed with profile: 95℃ for 45sec, 60℃ for 45sec, 72℃ for 45sec.

The final extension time given to the PCR reaction was 10min at 72℃ and storage at 4℃ for infinite time.

A final concentration of 2%TAE agarose gel with Ethidium bromide as a stain was made for running 10ul of PCR product mixed with 5ul of 1X loading dye along with 100bp DNA ladder in an electrophoresis tank. Wealtec Dolphin Doc (S/N470883) was later used to visualize the gel. Band sized of GAPDH was confirmed after comparison with the DNA ladder.

3.7.2 Optimization of VEGF gene:

Following set up for PCR reaction was used:

Taq buffer_____2ul

2.5mM MgCl2_____2.8ul

2mM dNTPs_____2ul

Forward primer_____2ul (20pmole)

Reverse primer_____2ul (20pmole)

cDNA_____ 5ul (approx 0.5ug)

Nuclease free water_____3.7ul

Taq Polymerase_____0.5ul (2.5unit)

Final reaction volume_____20ul

PCR machine used for carrying out the experiment was 9700 Applied Biosystem thermocycler. PCR profile used was preheating the reaction mixture at 95℃ for 5min, later 35 cycles were performed with profile: 95℃ for 45sec, 60℃ for 45sec, 72℃ for 45sec. The final extension time given to the PCR reaction was 10min at 72℃ and storage at 4℃ for infinite time.

A final concentration of 2%TAE agarose gel with Ethidium bromide as a stain was made for running 10ul of PCR product mixed with 5ul of 1X loading dye along with 100bp DNA ladder in an electrophoresis tank. Wealtec Dolphin Doc (S/N470883) was later used to visualize the gel. Band sized of VEGF was confirmed after comparison with the DNA ladder.

3.7.3 Optimization of TGF-β and NFκB:

Following set up for PCR reaction was used:

Taq buffer_____2ul

2.5mM MgCl2_____3ul

10mM dNTPs_____1ul

Forward primer_____1ul (10pmole)

Reverse primer_____1ul (10pmole)

cDNA_____ 5ul (approx 0.5ug)

Nuclease free water_____6.5ul

Taq Polymerase_____0.5ul (2.5unit)

Final reaction volume_____20ul

PCR machine used for carrying out the experiment was 9700 Applied Biosystem thermocycler. PCR profile used was preheating the reaction mixture at 95℃ for 5min, later 35 cycles were performed with profile: 95℃ for 45sec, 60℃ for 45sec, and 72℃ for 45sec. The final extension time given to the PCR reaction was 10min at 72℃ and storage at 4℃ for infinite time.

A final concentration of 2%TAE agarose gel with Ethidium bromide as a stain was made for running 10ul of PCR product mixed with 5ul of 1X loading dye along with 100bp DNA ladder in an electrophoresis tank. Wealtec Dolphin Doc (S/N470883) was later used to visualize the gel. Band size of TGFβ and NFκB was confirmed after comparison with the DNA ladder.

3.7.4 Optimization of PI3K:

Following set up for PCR reaction was used:

Taq buffer_____1.5ul

2.5mM MgCl2_____1.2ul

10mM dNTPs_____0.5ul

Forward primer_____1ul (10pmole)

Reverse primer_____1ul (10pmole)

cDNA_____ 5ul (approx 0.5ug)

Nuclease free water_____9.3ul

Taq Polymerase_____0.5ul (2.5unit)

Final reaction volume_____20ul

PCR machine used for carrying out the experiment was 9700 Applied Biosystem thermocycler. PCR profile used was preheating the reaction mixture at 95℃ for 5min, later 35 cycles were performed with profile: 95℃ for 45sec, 58℃ for 45sec, and 72℃ for 45sec. The final extension time given to the PCR reaction was 10min at 72℃ and storage at 4℃ for infinite time.

A final concentration of 2%TAE agarose gel with Ethidium bromide as a stain was made for running 10ul of PCR product mixed with 5ul of 1X loading dye along with 100bp DNA ladder in an electrophoresis tank. Wealtec Dolphin Doc (S/N470883) was later used to visualize the gel. Band size of PI3K was confirmed after comparison with the DNA ladder.

3.7.5 Optimization of AKT:

Following set up for PCR reaction was used:

Taq buffer_____1.5ul

2.5mM MgCl2_____1.2ul

10mM dNTPs_____0.5ul

Forward primer_____1ul (10pmole)

Reverse primer_____1ul (10pmole)

cDNA_____ 5ul (approx 0.5ug)

Nuclease free water_____9.3ul

Taq Polymerase_____0.5ul (2.5unit)

Final reaction volume_____20ul

PCR machine used for carrying out the experiment was 9700 Applied Biosystem thermocycler. PCR profile used was preheating the reaction mixture at 95℃ for 5min, later 35 cycles were performed with profile: 95℃ for 45sec, 57℃ for 45sec, and 72℃ for 45sec. The final extension time given to the PCR reaction was 10min at 72℃ and storage at 4℃ for infinite time.

A final concentration of 2%TAE agarose gel with Ethidium bromide as a stain was made for running 10ul of PCR product mixed with 5ul of 1X loading dye along with 100bp DNA ladder in an electrophoresis tank. Wealtec Dolphin Doc (S/N470883) was later used to visualize the gel. Band size of AKT was confirmed after comparison with the DNA ladder.

3.8 Optimization of Real Time PCR amplification:

All the genes were optimized for real time PCR under following mentioned conditions and PCR profiles. cDNA concentration used was 500ng. Following was the setup used for carrying out the PCR reaction:

Maxima® SYBER green/ ROX qPCR Master Mix (2X)_____ 12.5ul

cDNA template_____ 500ng

Forward Primer_____ 0.75ul (0.3uM)

Reverse Primer _____ 0.75ul (0.3uM)

Nuclease free Water _____ upto 25ul

Total volume _____ 25ul

PCR machine used for carrying out the experiment was 7300 Applied Biosystem Real time PCR. PCR profile used was preheating the reaction mixture at 95℃ for 10min, later 40 cycles were performed with profile: 95℃ for 15sec, 60℃ for 60sec.

A final concentration of 2%TAE agarose gel with Ethidium bromide as a stain was made for running 10ul of PCR product mixed with 5ul of 1X loading dye along with 100bp DNA ladder in an electrophoresis tank. Wealtec Dolphin Doc (S/N470883) was later used to visualize the gel. Band size of all the genes were confirmed after comparison with the DNA ladder.

3.9 Calculations of Real time PCR results:

The Ct values of both the target genes and the reference genes (housekeeping) were observed and compared to determine the fold change expression difference of the targeted markers in platelets of HCC patients. Formula used for calculating the change is as follows:

Ct value of the reference gene in control sample = A1

Ct value of the reference gene in patient sample = A2

Ct value of the target gene in control sample = B1

Ct value of the target gene in Patient sample = B2

ΔCt control sample = (Ct of target gene in control) – (Ct of reference gene in control) = B1- A1 = ΔCt1

ΔCt Exp = (Ct of target gene in patient sample) – (Ct of reference gene in Experiment) = B2 – A2 = ΔCt2

ΔΔCt = (ΔCt of Patient) – (ΔCt of control) = ΔCt2- ΔCt1

Fold change (FC) = $2^{(-\Delta\Delta Ct)}$

3.10 Statistical Analysis:

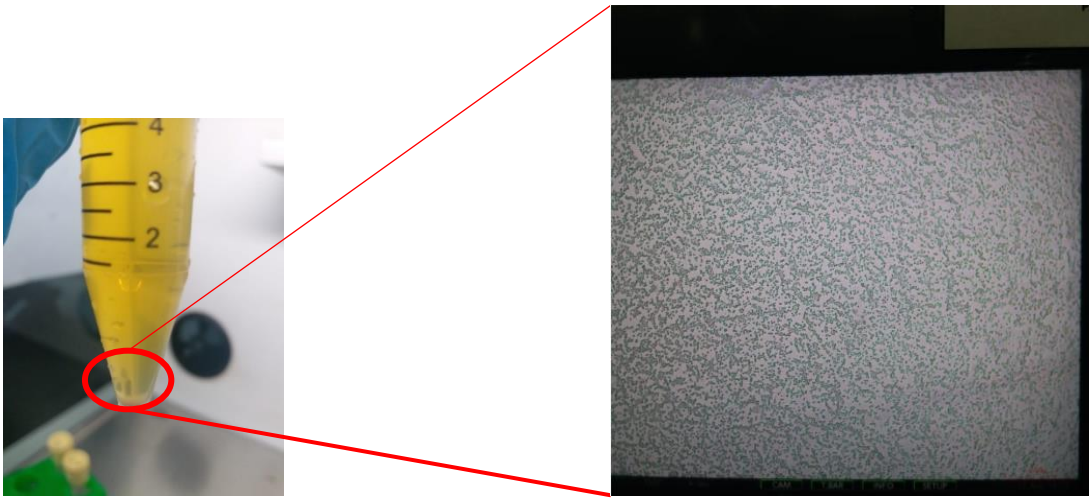
Graph pad prism version 7.0 was used for carrying out all of the statistical tests. Hypothesis was tested by using one-way ANOVA. *P<0.05, **P<0.005, ***P<0.001 was considered significant with Confidence interval = 95%.

Chapter 4

RESULTS

4.1 Platelets Extraction:

The blood samples of HCC patients obtained from Holy family hospital were subjected to platelet extraction via centrifugation. A white pallet of platelets was obtained after the successful centrifugation. Purity of the platelets was confirmed by visualizing the platelets under the microscope. Presence of 3 or 4 white blood cells on the background of millions of platelets were considered to be 99.9% pure and hence ready to be used for RNA extraction.



(a) **(b)**

Figure 4.1: Confirmation of integrity of platelets extracted via centrifugation: **(a)** depicts the platelets pallet extracted via centrifugation. **(b)** Platelets under the microscope showing their inactive state**.**

4.2 Determining purity of platelets:

The obtained platelet pallet was subjected to FACS analysis for identification. FITC conjugated CD151 antibody stained platelets were detected at the FL1 detector as shown in the figure below:

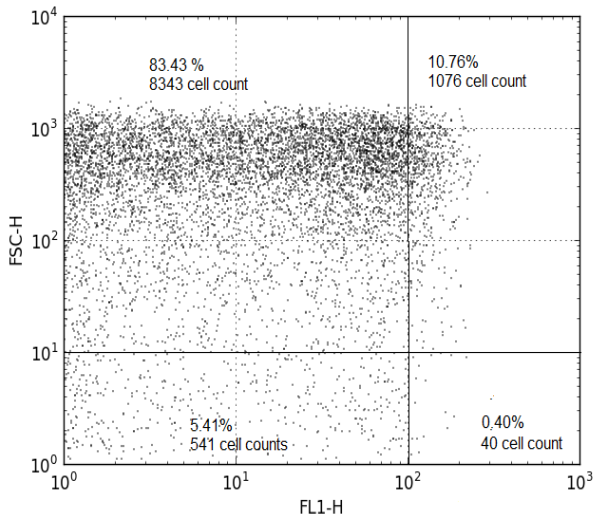


Figure 4.2: Confirmation of integrity of platelets extracted via FACS analysis: Graphical representation shows the detection of FITC conjugated CD151 antibody stained platelets in the gated area.

4.3 RNA Extraction and cDNA synthesis:

After getting the pure population of platelets they were further proceeded for total RNA extraction. MagMax Rna isolation kit was used for this purpose. The quantity of RNA was calculated using spectrophotometer. The extracted RNA was converted into cDNA with

the use of oligo dT primers via RT-PCR. cDNA integrity was determined by amplification of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) a house keeping gene.

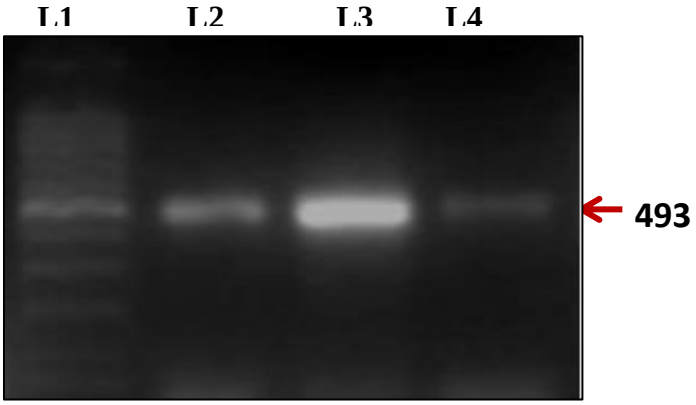


Figure 4.3: Digital image showing the amplification of GAPDH PCR (493bp) hence confirming cDNA synthesis. The clear bands of 493bp were observed in all samples in L2, L3 and L4 lane along with 100bp DNA ladder in Lane 1.

4.4 PCR Amplification of Selected Biomarkers reportedly known in Cancer Initiation, Progression and Metastasis

4.4.1 Optimization by Conventional PCR

TGFβ, NFkβ, VEGF, AKT and PI3K were selected as potential biomarkers due to their known ability for causing cancer initiation, progression and metastasis. For evaluating the expression of cellular genes about 500ng of the synthesized cDNA was used as a template for successful amplification of the selected genes in order to optimize them on conventional

PCR (Applied Biosystem PCR GeneAmp 9700 Thermocycler). After optimizing PCR profiles of all the genes, the experiment was repeated for 3 times for confirming the consistency and reproducibility of the results. No non-specific amplification was observed on gel.

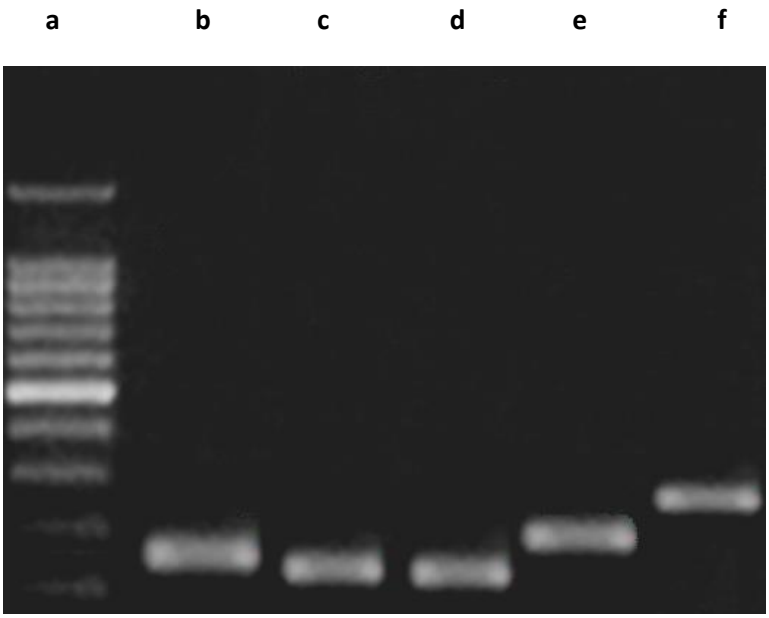


Figure 4.4: Gel electrogram of optimised set of primers using cDNA as template. **(a)** Lane shows 100 bp ladder. **(b)** Lane depicts band of VEGF of 173bp (**c)** Lane depicts NFkB amplicon of 155 bp (**c)** Lane depicts PI3K amplicon of 153 bp **(d)** Lane depicts AKT amplicon of 188 bp. **(e)** Lane depicts NFkB amplicon of 155 bp. **(f)** Lane depicts 240bp amplicon of TGFβ.

4.4.2 Quantitative Real Time PCR Amplification of Selected Bio-Markers.

After successful optimization of all the selected genes on conventional PCR they were optimized on Quantitative real time PCR (Applied Biosystems 7300 Real-Time PCR

system) by using 300ng of the cDNA as the template. Normalization of the amplification plots was performed with B- Actin for all the genes previously optimized on conventional PCR for both the control and the patient samples. For determining any secondary amplification, the dissociation curves for all the genes were analyzed.

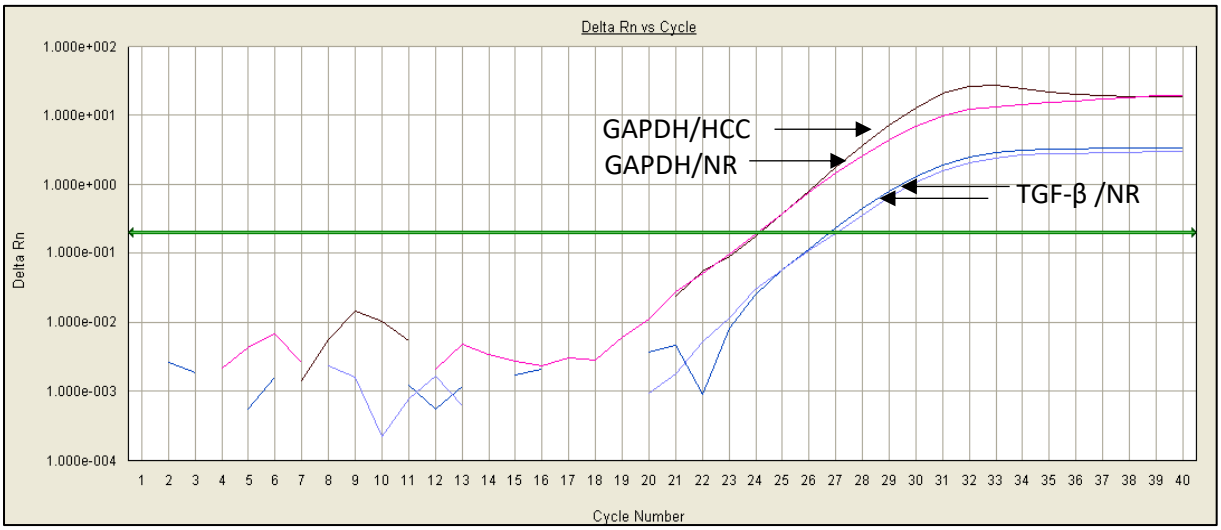


Figure 4.5: Data analysis of qRT-PCR: Delta Rn vs. Cycle Number. Amplification of GAPDH and TGF-β in control samples (NR) represented by qRT-PCR amplification plot.

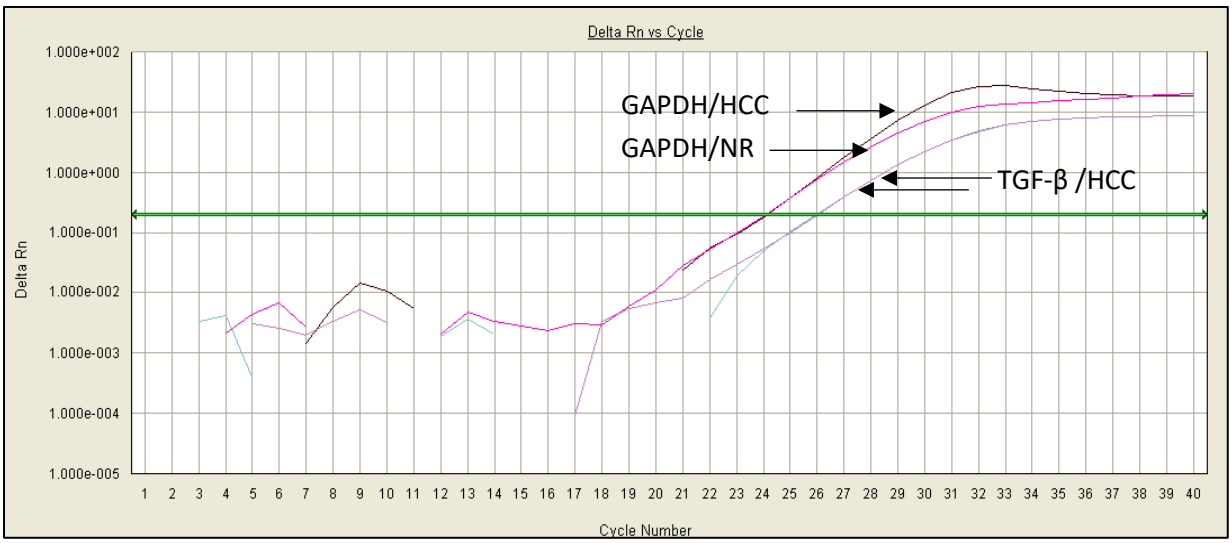


Figure 4.6: Data analysis of qRT-PCR: Delta Rn vs. Cycle Number. Amplification of GAPDH and TGF-β in HCC samples represented by qRT-PCR amplification plot.

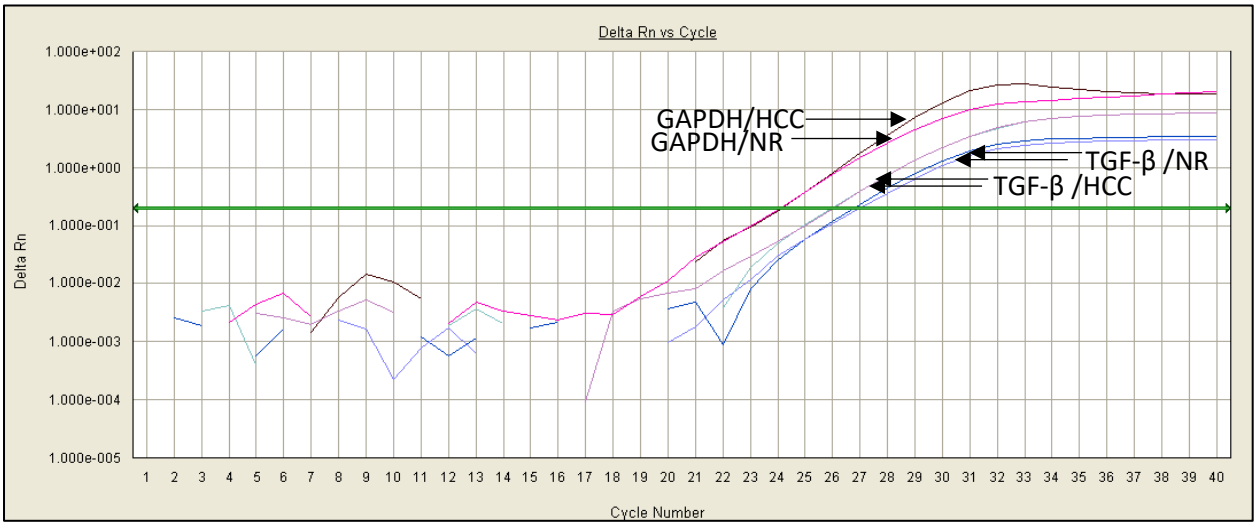


Figure 4.7: Data analysis of qRT-PCR: Delta Rn vs. Cycle Number. Amplification of GAPDH and TGF-β in control and HCC samples represented by qRT-PCR amplification plot.

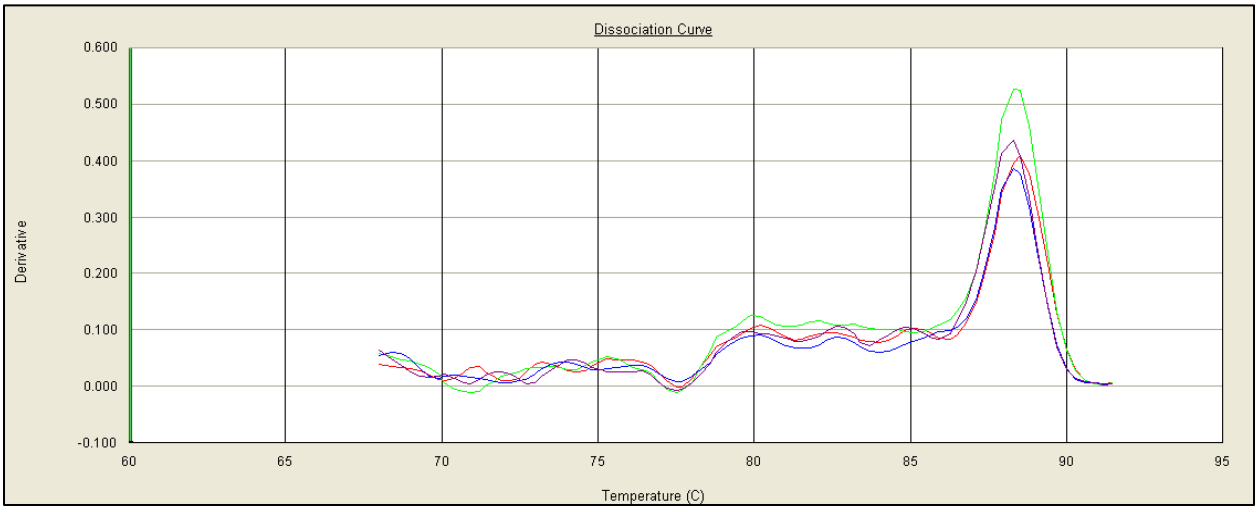


Figure 4.8: Data analysis of qRT-PCR: Dissociation curve of TGF-β in control and HCC samples depicting absence of secondary amplification and primer specificity.

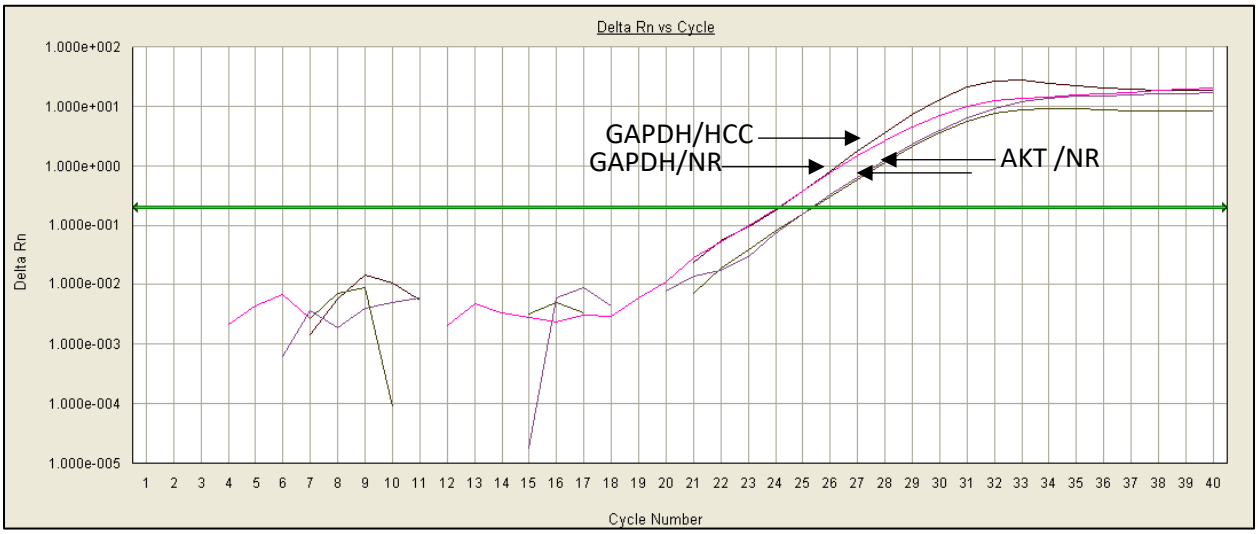


Figure 4.9: Data analysis of qRT-PCR: Delta Rn vs. Cycle Number. Amplification of GAPDH and AKT in control samples (NR) represented by qRT-PCR amplification plot.

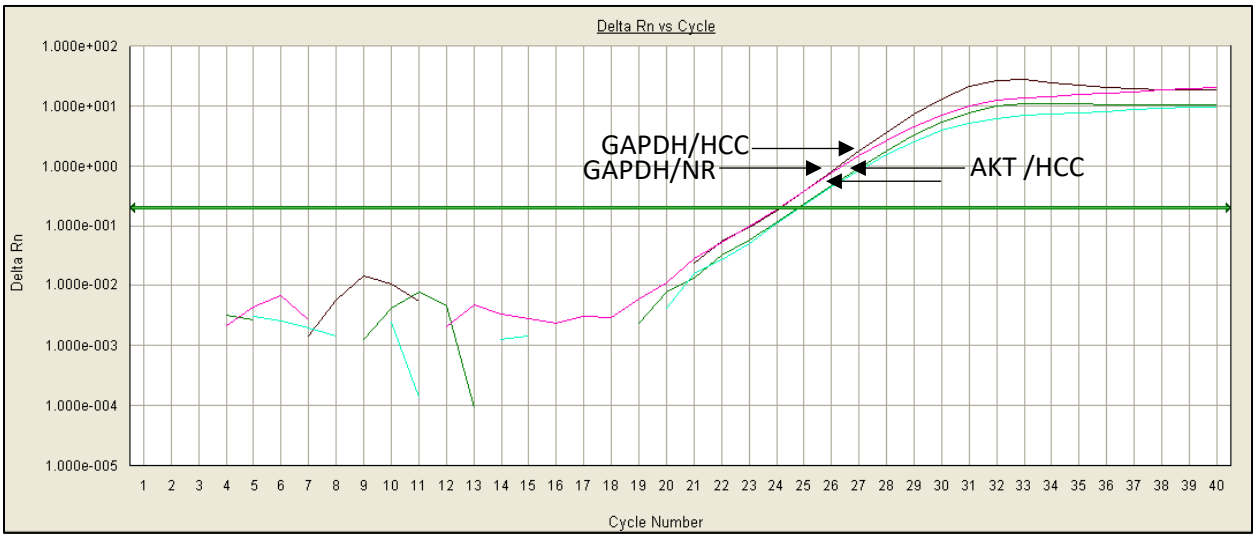


Figure 4.10: Data analysis of qRT-PCR: Delta Rn vs. Cycle Number. Amplification of GAPDH and AKT in HCC samples represented by qRT-PCR amplification plot.

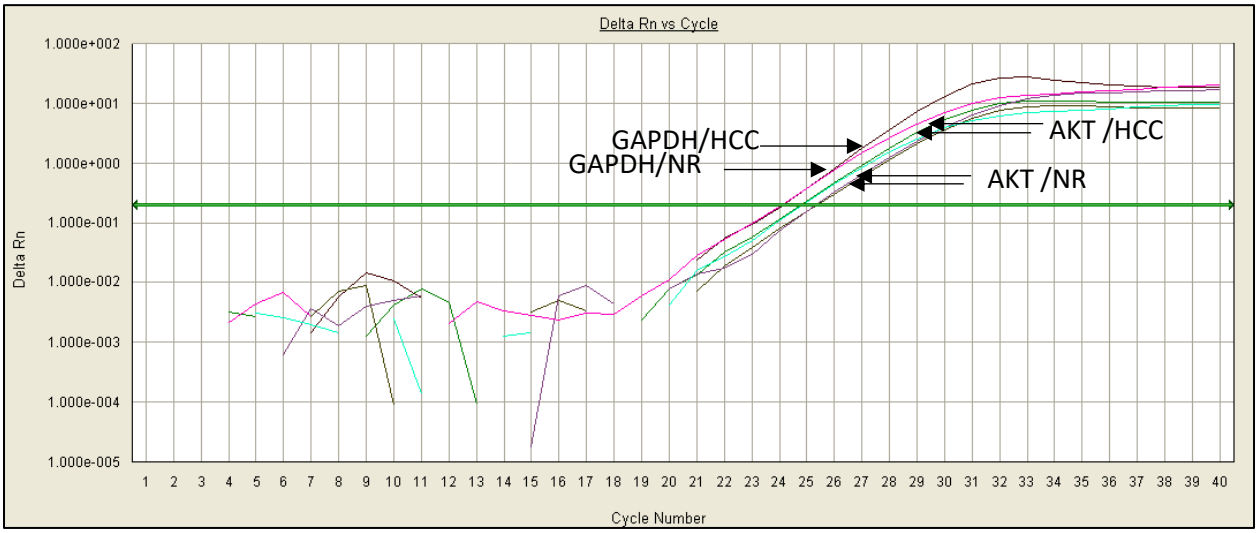


Figure 4.11: Data analysis of qRT-PCR: Delta Rn vs. Cycle Number. Amplification of GAPDH and TGF-β in control and HCC samples represented by qRT-PCR amplification plot.

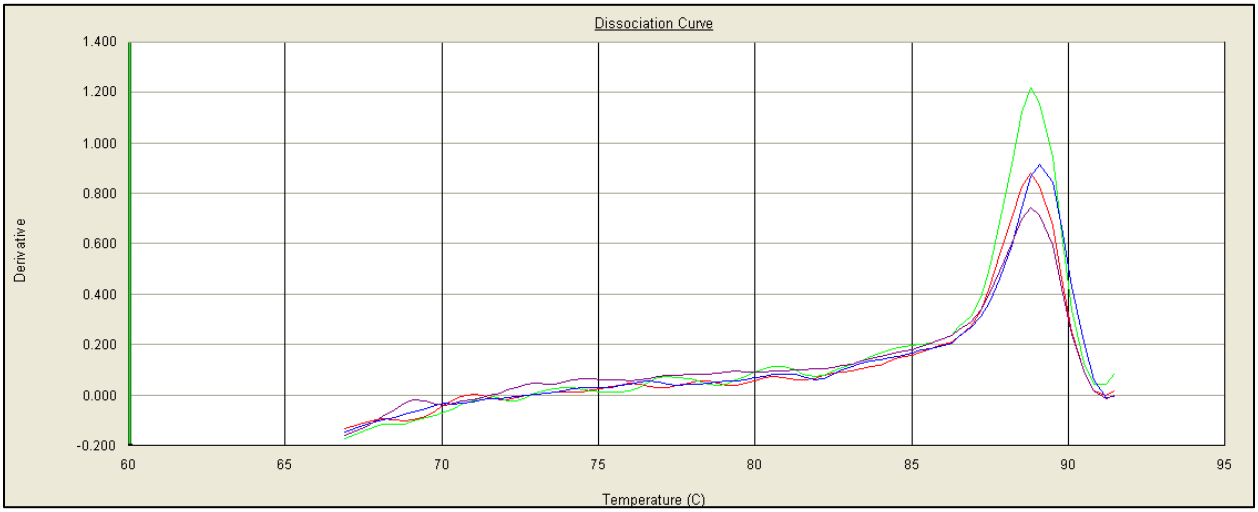


Figure 4.12: Data analysis of qRT-PCR: Dissociation curve of AKT in control and HCC samples depicting absence of secondary amplification and primer specificity.

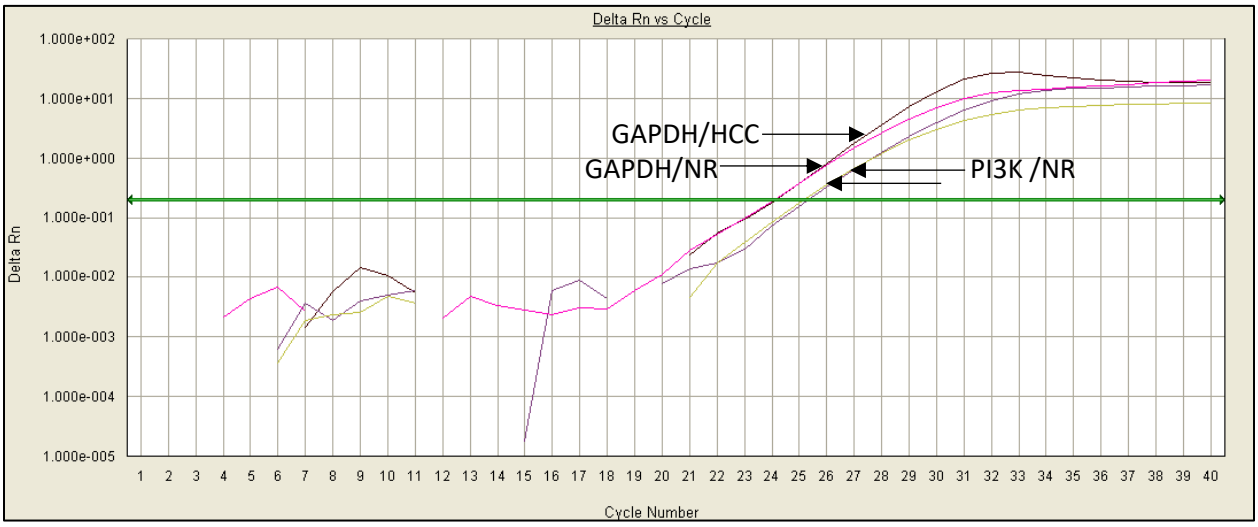


Figure 4.13: Data analysis of qRT-PCR: Delta Rn vs. Cycle Number. Amplification of GAPDH and PI3K in control samples (NR) represented by qRT-PCR amplification plot.

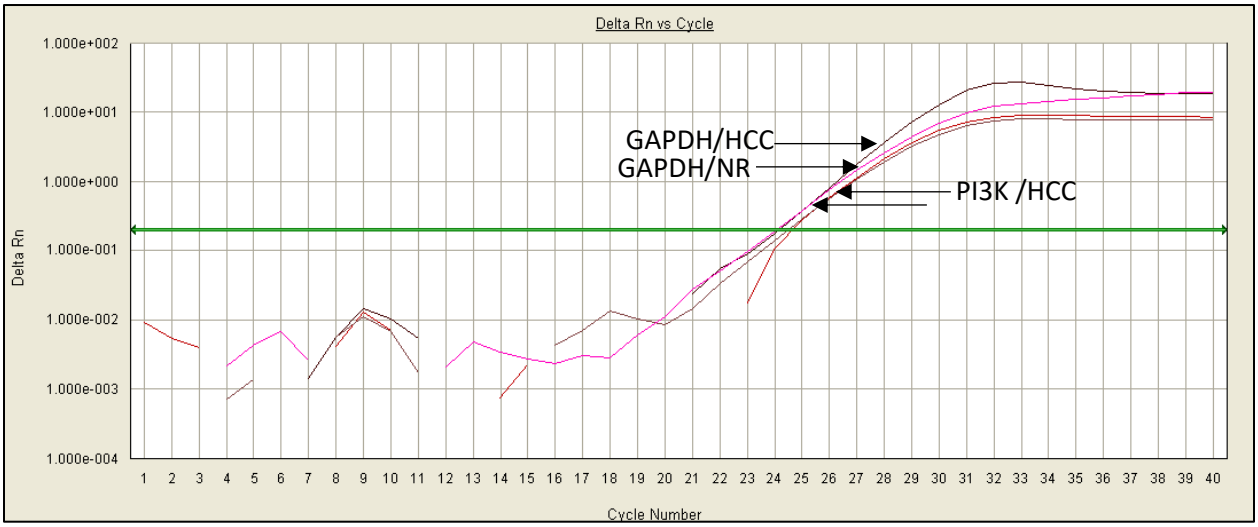


Figure 4.14: Data analysis of qRT-PCR: Delta Rn vs. Cycle Number. Amplification of GAPDH and PI3K in HCC samples represented by qRT-PCR amplification plot.

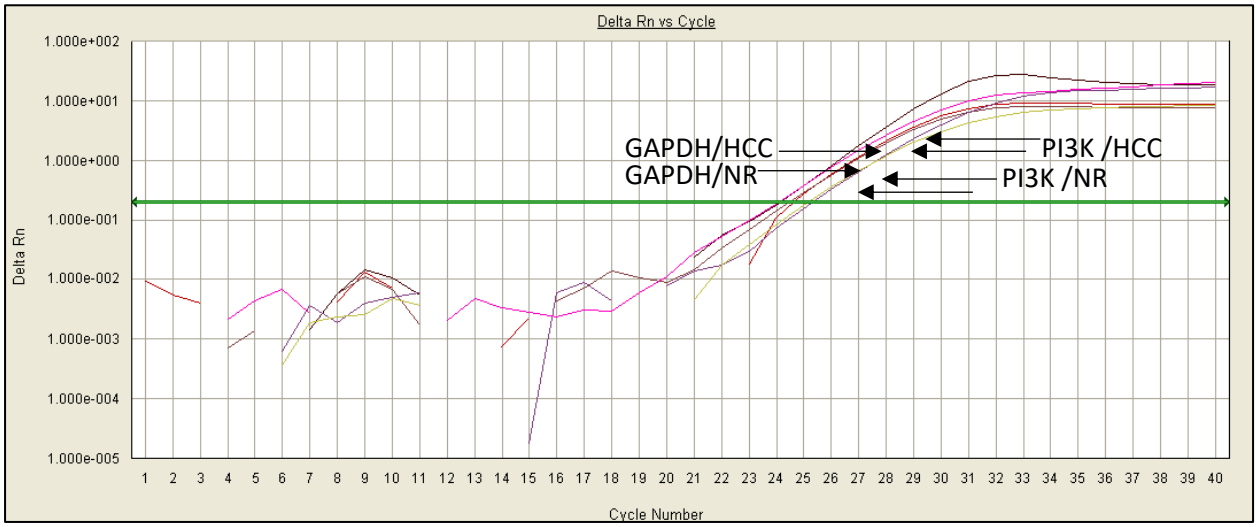


Figure 4.15: Data analysis of qRT-PCR: Delta Rn vs. Cycle Number. Amplification of GAPDH and PI3K in control and HCC samples represented by qRT-PCR amplification plot.

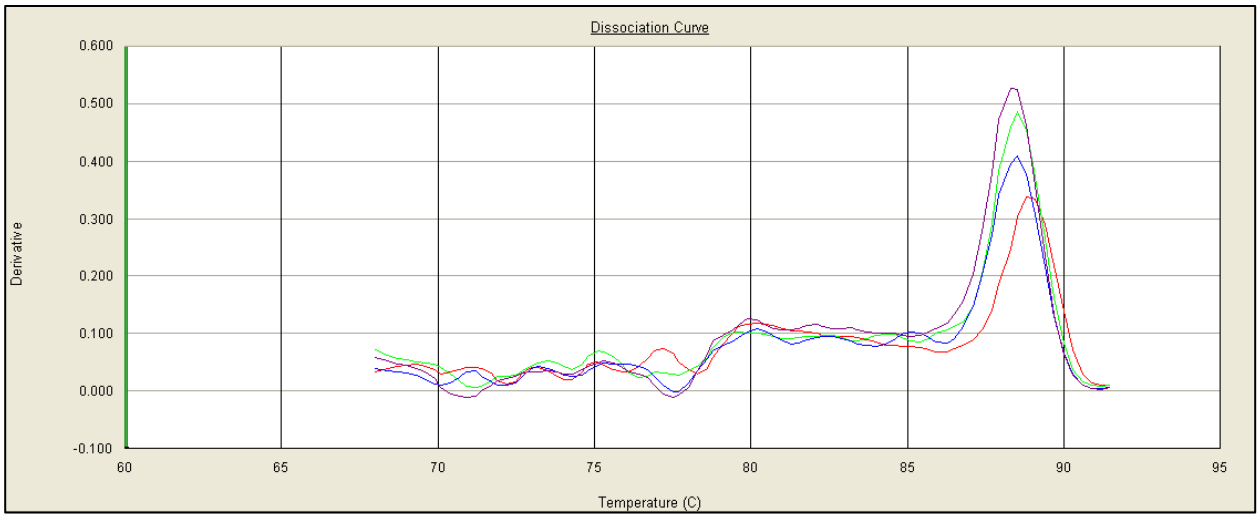


Figure 4.16: Data analysis of qRT-PCR: Dissociation curve of PI3K in control and HCC samples depicting absence of secondary amplification and primer specificity.

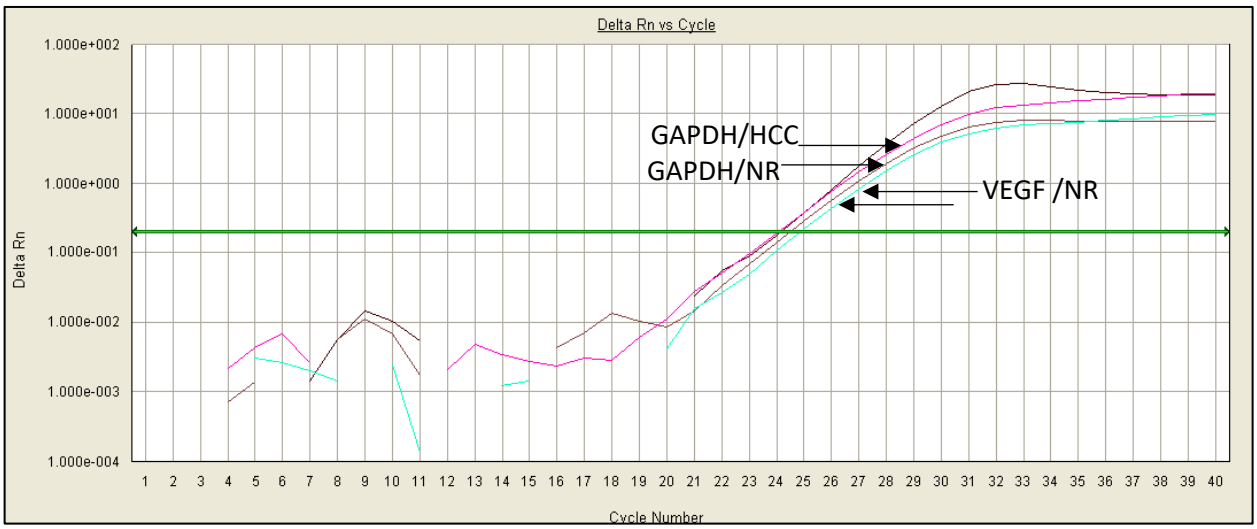


Figure 4.17: Data analysis of qRT-PCR: Delta Rn vs. Cycle Number. Amplification of GAPDH and VEGF in control samples (NR) represented by qRT-PCR amplification plot.

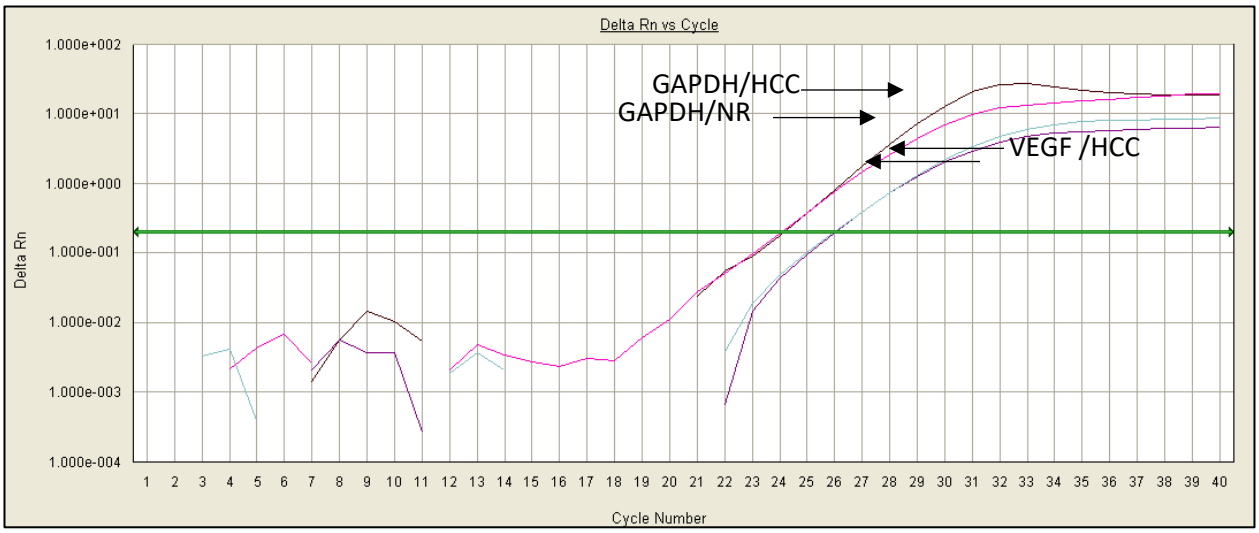


Figure 4.18: Data analysis of qRT-PCR: Delta Rn vs. Cycle Number. Amplification of GAPDH and VEGF in HCC samples represented by qRT-PCR amplification plot.

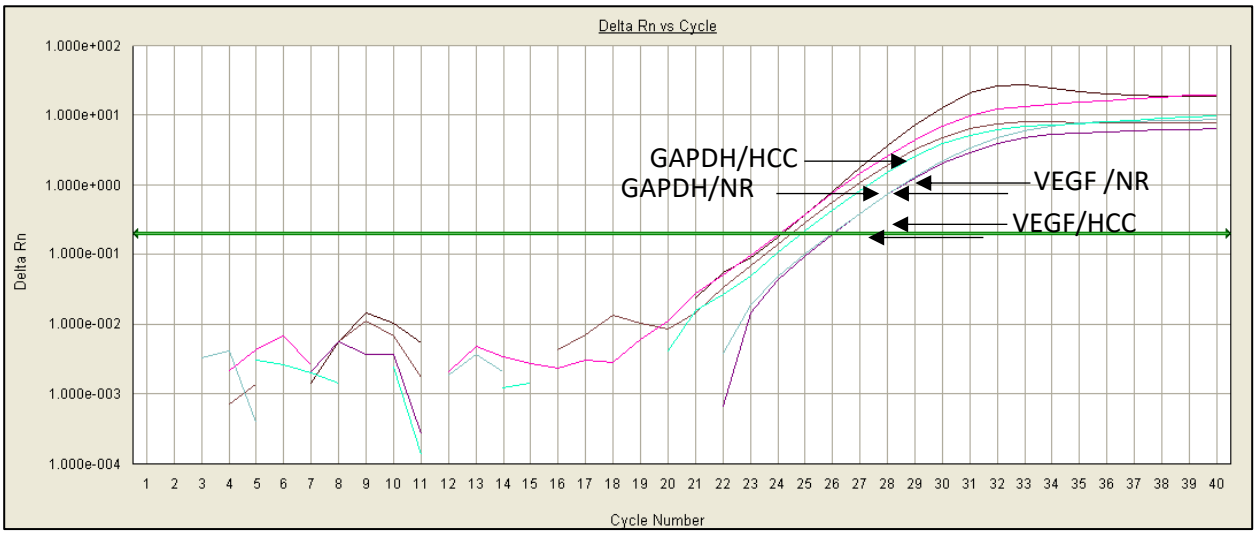


Figure 4.19: Data analysis of qRT-PCR: Delta Rn vs. Cycle Number. Amplification of GAPDH and VEGF in control and HCC samples represented by qRT-PCR amplification plot.

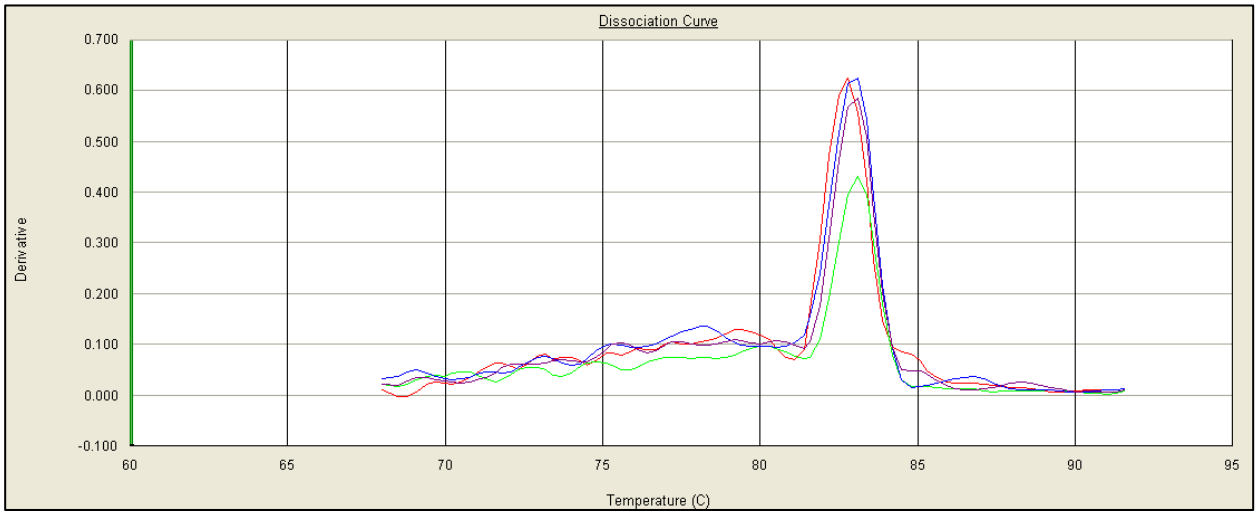


Figure 4.20: Data analysis of qRT-PCR: Dissociation curve of VEGF in control and HCC samples depicting absence of secondary amplification and primer specificity.

4.5 Gene expression analysis of Cancer markers

For identification of genes that can be potentially used as a biomarker for early liver cancer detection TGFβ, NFκB, AKT, PI3K and VEGF were selected due to their well-known role in cell initiation, progression and metastasis. The fold change in expression of these genes was recorded in healthy controls and patients. The mRNA expression of Beta Actin was used as an internal control. The expression of all the selected genes were obtained from individual experiments with replicates from each sample. Results obtained from the experiments are illustrated below:

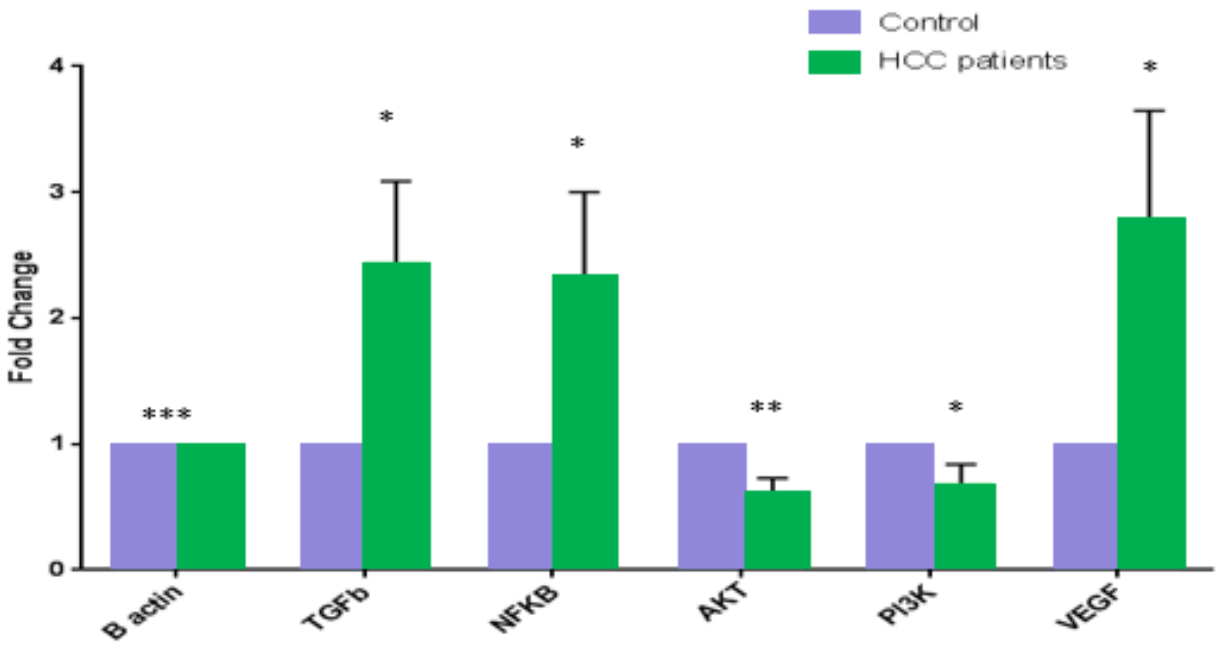


Figure 4.21: Expression level of TGF-β, NF-κβ, AKT, PI3K and VEGF in Control and HCC Patients

The figure describes the transcriptional analysis of all the selected genes. It depicts that in the platelets of HCC patients, the expression level of TGF-β, NF-κβ, and VEGF was significantly increased by 2.48, 2.35 and 2.78 folds respectively, whereas a decrease of 0.6 and 0.65 folds was observed in AKT and PI3K respectively as compared to control. Data was analyzed by applying student t-test. Significance: *P value < 0.05, **P value <0.005, ***P value < 0.001.

4.6 ROC Curve Analysis of Selected Biomarkers

Receiver operating characteristic curve or ROC curve is used for determining the diagnostic ability of a selected biomarker. The false positive rate or the specificity is plotted on X axis. The true positive rate or sensitivity is plotted on y axis. The potential of selected biomarkers to successfully detect HCC cancer patients from healthy controls was determined by ROC curve analysis. Furthermore, ROC curve analysis was performed to determine the potential of all the selected biomarkers for detecting early and late stage HCC. Roc curve analysis of AKT and PI3K showed that these biomarkers were able to detect early stage HCC with more specificity than any other biomarker.:

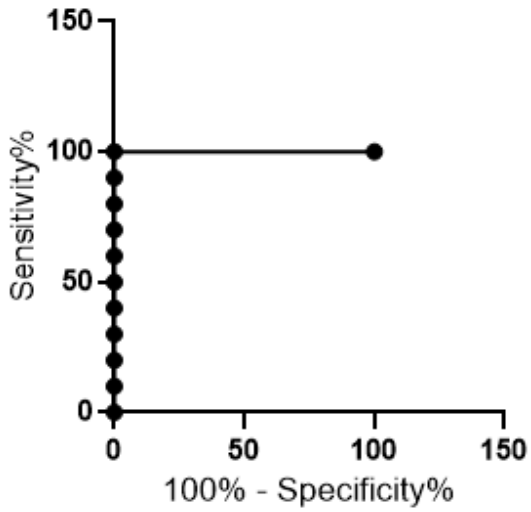


Figure 4.22: ROC curve analysis of TGF-β, NF-κβ, AKT, PI3K and VEGF: ROC curve analysis showed that all the biomarkers detected HCC with 100% sensitivity. AUC = 1.00

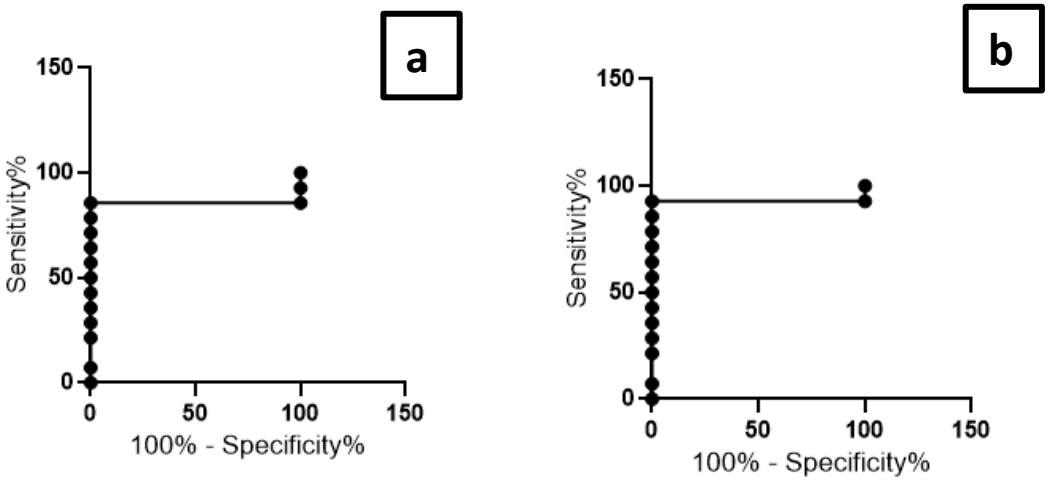


Figure 4.23: ROC curve analysis of AKT and PI3K: (a) The ROC curve analysis depicts that PI3K was able to detect early stage HCC with AUC= 0.9286. (b) ROC curve analysis showed that AKT was able to detect early stage HCC with AUC= 0.8751.

4.7 Analysis of the Expression pattern in patients with different HCC Stages

4.7.1 Expression of TGF-β, NFkβ, VEGF, AKT and PI3K in patients with various stages of HCC:

The expression of TGF-β, NFkβ, VEGF, AKT and PI3K at various stages of HCC was determined. The expression analysis determined a definitive change in expression pattern of all these biomarkers as compared to controls.

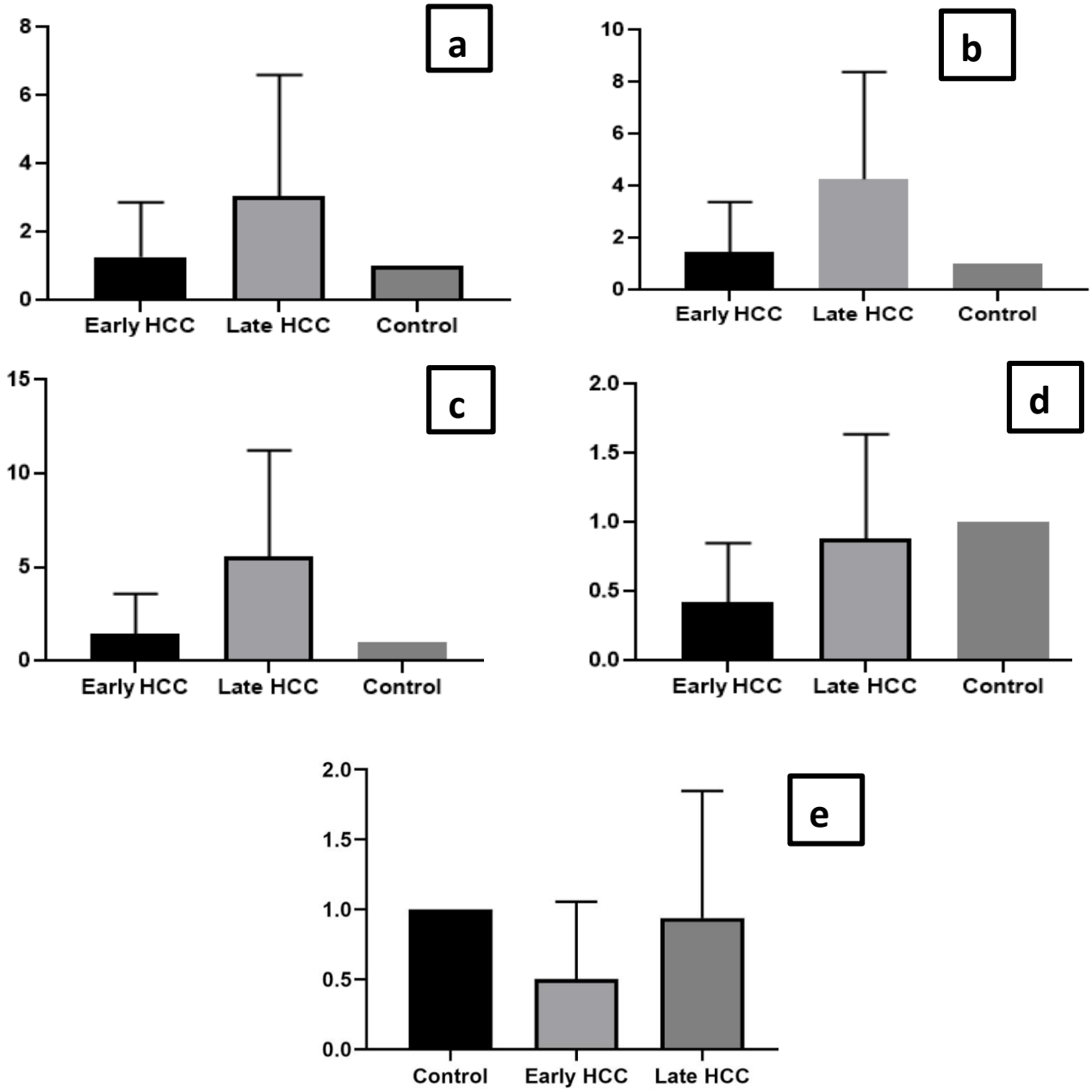


Figure 4.24: Graphical analysis showing the expression of selected biomarkers in detecting early and late stage HCC: **(a)** TGF-β was observed to be increased in all stages of HCC. **(b)** NF-κB was observed to be increased in all stages of HCC. **(c)** VEGF was observed to be increased in all stages of HCC. **(d)** Expression of AKT was observed to be decreased in all stages of HCC. **(e))** PI3K was observed to be decreased in all stages of HCC

Chapter 5

DISCUSSION

A characteristic abnormal growth of cells with a potential to invade the surrounding is known as cancer. Cancer can be of many types depending upon its place of origin. Cancer that originates in the liver organ is called Liver Cancer. Among several types of liver cancers, Hepatocellular carcinoma is the most prevalent type. There is a constant rise observed in the reported HCC cases each year due to several contributing factors. One of the contributing factors for HCC prevalence is late diagnosis of the HCC. HCC patients are usually diagnosed at a very late stage when they are left with zero to no treatment options.

Current available choices for detecting tumor include tissue biopsy and imaging techniques i.e. CT scan. However due to its limitation of being invasive in nature there is a need to explore other diagnostic measures. Liquid biopsy due to its promising results is emerging as a powerful tool for diagnosis of cancer. Platelets interact with tumor directly or indirectly causing alteration in mRNA repertoire of platelets. These changed mRNA can be exploited for determining a specific biomarker for diagnosis of HCC at an early stage as determined by related studies conducted in other types of cancers i.e. Lung and hepatobiliary cancer (Best et al., 2015).

The present study was designed to explore the potential of the selected biomarkers for early diagnosis of HCC using liquid biopsy samples. The goal of our study was to establish a noninvasive, cost effective and reliable way for early detection of HCC. For this purpose, transcriptional analysis of all the selected biomarkers i.e. TGF-β, NFκβ, VEGF, AKT and

PI3K was performed. The results depicted that TGF-β, NFκβ, and VEGF showed a significant increase of 2.48, 2.35 and 2.78 folds respectively. All these biomarkers are well known for their pro-tumor effect (Quintanilla, Castillo, Kocić, & Santibañez, 2012) hence, an increase in their expression level indicated their probable role in assisting tumor initiation, development and dissemination. Higher expression level of TGF-β may cause an increase in the release of TGF-β molecules from platelets. These increased TGF-β molecules may bind to their respective receptors on the tumor cells and assist the tumor in its growth (J. J. Huang & Blobe, 2016). Apart from tumor proliferation TGF-β also helps in inflammation hence assisting in fibrosis and ultimately helping in HCC development. TGF-β also contributes in tumor metastasis as it is involved in causing cell motility and invasion via EMT (Epithelial mesenchymal transition) (Liu & Desai, 2015).. TGF-β is not only involved in proliferation of tumor but also in survival of tumor cell as it also inhibits the apoptosis (J. J. Huang & Blobe, 2016). The results of our study are in accordance with the previous findings of Best et al., 2015 in hepatobiliary cancer, pancreas Adenocarcinoma, breast cancer and colorectal cancer.

An increase in NFκβ expression in HCC patients was observed in our study as it is in agreement with the previously reported findings of Best et al., 2015 in Non Small cell lung carcinoma. An increase in NFκβ within platelets may change certain pathways in the platelets. Owing to the increased expression of NFκβ in the platelets, they would be able to bind with fibrinogen more effectively. This binding of platelets with fibrinogen starts the cascade of events that lead towards the activation of platelets. Moreover, the increased expression of NFκβ also helps in better interaction between platelets and tumor cells. This binding of platelets with the tumor cell not only helps in activation of platelets but also

helps in cloaking the tumor cell from immune surveillance (Fuentes et al., 2016). Elevated levels of NFκβ also helps in activation of platelets directly by causing the release of granules out of the platelets.

These granules contain molecules that further promote the tumor cell survival, proliferation and metastasis. One such molecule released is VEGF. This molecule is well known for initiating angiogenesis (Carmeliet, 2005). A tumor during its growth requires nutrition which is provided by the nearby blood vessels. As the tumor increases in size for meeting its nutrition requirements it starts to build blood vessels around itself. The signals for the blood vessel formation is usually taken by VEGF molecule. VEGF not only assist proliferation of tumor at its primary site of origin but also helps in its proliferation at the secondary site by initiating angiogenesis at that site. This provides the tumor with its required nutrition needed for survival at secondary site. Interestingly an increase in VEGF molecule was also observed during the transcriptional analysis in Platelets of HCC patients. Hence, the increase in fold change of VEGF also indicates the positive role in tumor growth and metastasis. This result of our study was in conformity of the findings reported by (Hui-HsinKo, et al., 2015) who studied the VEGF mRNA level in oral squamous cell carcinoma tissue (OSCC). The researcher also concluded that the study of VEGF mRNA levels can be used for prognosis and progression of OSCC.

The expression of both selected biomarkers i.e. AKT and PI3k was seen to be decreased. Although both AKT and PI3K are known to have pro tumor effect as they inhibit apoptosis and help in cell proliferation and survival as well as metastasis through initiation of EMT (Epithelial mesenchymal transition). In platelets usually, the binding of thrombin molecule with its respective receptors on the surface of platelets initiate the PI3K/AKT pathway.

This initiation of pathway then leads to platelets activation which causes the release of granules from the platelets. These granules released contain many molecules that have pro tumor effect and they promote tumor proliferation as well as cause further activation of platelets and the cycle continues (Woulfe et al., 2004). But on the contrary a decrease in the expression of AKT and PI3K was observed in our experiment. This decrease in the expression could be due to various reasons which include the specific degradation of mRNA population phenomenon within platelets. There might be certain events going on during the interaction of platelets with HCC that cause the specific degradation of the mRNA of AKT and PI3K in the platelets. Translational analysis should be performed to further explore it.

Receiver operating characteristic curve or ROC curve is used for determining the diagnostic ability of a selected biomarker. The false positive rate or the specificity is plotted on X axis. The true positive rate or sensitivity is plotted on y axis. The potential of selected biomarkers to successfully detect HCC cancer patients from healthy controls was determined by ROC curve analysis. Furthermore, ROC curve analysis was performed to determine the potential of all these biomarkers for detecting early and late stage HCC. The ROC curve analysis of all the biomarkers depicted that all the biomarkers were able to detect HCC with 100% sensitivity. Furthermore, the expression of all the selected biomarkers in HCC patient was also related to the different stages of HCC. TGF-β was seen to increase with the increase in stage from early to late stage of HCC as compared to the control. NF-κB expression was also observed in HCC patients with different stages of HCC. The same trend as that of TGF-β was observed in NFκβ. An increase in expression

was observed with the increase in stage of HCC. VEGF gene expression was also observed to be increased in all stages of HCC along with the expression of TGF-β and NFκβ.

On the contrary expression of AKT was observed to be decreased in all stages of HCC as compared to controls with an increase in expression with advancement of HCC stage. ROC curve analysis of AKT expression showed that it was able to detect early stage HCC with AUC= 0.8751.

Similar trend was observed in case of PI3K as it was seen to be decreased in all stages of HCC from the control but the expression of PI3K was seen to increase with the advancement in stages of HCC. ROC curve analysis showed that PI3K was able to detect early stage HCC with AUC= 0.9286.

The results of our study are quite promising as all the selected biomarkers successfully detect HCC patients. However, AKT and PI3K were able to detect early stage HCC with higher significance as compared to other selected biomarkers. Potential of these biomarkers for differentiating the HCC stages should be verified by carrying out the study on a larger scale

CONCLUSION AND FUTURE PROSPECTS

The outcomes of our study depict that all the selected biomarkers can be used for the detection of HCC. Furthermore, the selected biomarkers i.e. AKT and PI3K, in comparison to other biomarkers i.e. TGF-β, NF-κB and VEGF in the study, carry better potential to be used for detection of early stage HCC as indicated by the results of the study. Present study could prove to be beneficial in determining the altered RNA levels in the platelets of HCC patients as compared to control and can be a step forward in establishing the altered mRNA profile to be used for early HCC detection.

However, further research should be conducted for identification of more biomarkers for HCC detection and stage differentiation. It is necessary that the translational levels of all the selected biomarkers are studied in detail along with the use of a combination of different biomarkers for the detection of HCC at an early stage at a larger scale.

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Appendix

50X TAE buffer

EDTA 18.6g

Tris base 121g

Adjust pH to 8.5 with glacial acetic acid. Adjust volume to 500ml with distilled water.

INFORMED CONSENT FORM

Analyzing the Tumor Educated Platelets (TEP) as Potential Bio-Marker in Molecular Diagnosis of Liver Cancer: A minimally Invasive Approach alternative to Tissue Biopsy

AIMS AND OBJECTIVES

Assessment of the mRNA repertoire obtained from isolated platelets of the patients as potential biomarkers for early detection of liver cancer

SAMPLE COLLECTION

10ml blood sample will be collected from all participants

INFORMED CONSENT

You are being asked to participate in a research study for early detection of liver cancer by using mRNA repertoire obtained from isolated platelets from blood samples. You will be asked to donate 10ml of blood. This will not cause any physical injury. Your samples will be preserved in the laboratory only for the purpose of this study. Your identity in this study will be protected. You can terminate your participation at any time in the course of this study. This research project will be carried out solely on a non-commercial basis for research proposes. Your participation in this study is voluntarily. Further, if it becomes necessary, in your interest, counselling will be provided. The scientific information will only be shared among the collaborating scientists. The results of the study if novel or of medical interest will be published in scientific journals without disclosing your identity

I hereby confirm that I fully understand what has been stated above. I voluntarily donate blood sample from myself/ and from my family for research purposes only.

Signature/Thumb impression of the patient:

_____

Name: _____ **Participant ID:**

_____

Contact no: _____

General Information of Participant

Age**:** _____ Gender: _____ Phone: _____

City: _____ Ethnicity: _____ Weight: _____

Occupation: _____ Diagnosis: _____

Comorbidities: _____

Sample ID: _____

Symptoms

_____
_____
_____
_____
_____

Stage of liver disease diagnosed:

_____
_____
_____
_____

Any therapy:

_____
_____
_____
_____
_____

Information required

CBC: _____ BMI: _____ Hemoglobin: _____

Platelets: _____ Spleen size: _____ Differential count: _____

Lymphocytes: _____ RBCs: _____ WBCs: _____

ALT: _____ AFT: _____ Viral load: _____

ASAB

ATTA-UR-RAHMAN SCHOOL OF APPLIED BIOSCIENCES
NATIONAL UNIVERSITY OF SCIENCES & TECHNOLOGY

Ref:No: 38 IRB-

Date: 25 April, 2016

IRB APPROVAL LETTER

Project Title: Analyzing the tumor educated platelets (TEP) as potential Bio-Marker in molecular diagnosis of Liver cancer: A minimally invasive approach alternative to tissue biopsy.

Name of Principal Investigator I: Dr. Sobia Manzoor

Name of Principal Investigator II: --

Field and Subfield of Project: Biosciences

Duration: 24 Month

Name of the Department: Healthcare Biotechnology, ASAB, NUST

The aforesaid project has been reviewed by Institutional Review Board (IRB) Committee, ASAB, keeping in view the following selection criteria:

- Qualification, Expertise and Scientific Caliber of the Principal Investigators
- Proposed Goals of the Study
- Subject Selection
- Selection Criteria of Subjects
- Informed Consent Process
- Potential Problems
- Research Design and Methods
- Potential Benefits of the Study
- Risks of the Study
- Management of Risks
- Assessment of Risk
- Confidentiality
- Conflict of Interest

The committee thus **APPROVES** the project on "**Analyzing the tumor educated platelets (TEP) as potential Bio-Marker in molecular diagnosis of Liver cancer: A minimally invasive approach alternative to tissue biopsy**" on the scales and criterion set by IRB.

Dr. Najam us Sahar Zaidi
Member, IRB
ASAB, NUST

Dr. Muhammad Tahir
Member, IRB
ASAB, NUST

Dr. Hajra Sadia
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Diagnostic Potential of Platelets Derived Selected Tumor Biomarkers in Hepatocellular Carcinoma

ORIGINALITY REPORT

3%	%	3%	%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Nik Sol, Thomas Wurdinger. "Platelet RNA signatures for the detection of cancer", Cancer and Metastasis Reviews, 2017 Publication	<1%
2	Best, Myron G., Nik Sol, Irsan Kooi, Jihane Tannous, Bart A. Westerman, François Rustenburg, Pepijn Schellen, Heleen Verschueren, Edward Post, Jan Koster, Bauke Ylstra, Najim Ameziane, Josephine Dorsman, Egbert F. Smit, Henk M. Verheul, David P. Noske, Jaap C. Reijneveld, R. Jonas A. Nilsson, Bakhos A. Tannous, Pieter Wesseling, and Thomas Wurdinger. "RNA-Seq of Tumor-Educated Platelets Enables Blood-Based Pan-Cancer, Multiclass, and Molecular Pathway Cancer Diagnostics", Cancer Cell, 2015. Publication	<1%
3	Cancer Metastasis – Biology and Treatment, 2010. Publication	<1%

4	"Antibody Therapy", Springer Nature, 2018 Publication	<1%
5	Kulkarni, Sunil A., and Tara S. Barton-Maclaren. "Performance of (Q)SAR Models for Predicting Ames Mutagenicity of Aryl Azo and Benzidine Based Compounds", Journal of Environmental Science and Health Part C, 2014. Publication	<1%
6	Farakh Javed, Sobia Manzoor. "HCV non-structural NS4A protein of genotype 3a induces mitochondria mediated death by activating Bax and the caspase cascade", Microbial Pathogenesis, 2018 Publication	<1%
7	Wataru Okajima, Shuhei Komatsu, Daisuke Ichikawa, Mahito Miyamae et al. "Liquid biopsy in patients with hepatocellular carcinoma: Circulating tumor cells and cell-free nucleic acids", World Journal of Gastroenterology, 2017 Publication	<1%
8	Zaczek, A.. "Clinical evaluation of developed PCR-based method with hydrolysis probes for TOP2A copy number evaluation in breast cancer samples", Clinical Biochemistry, 201007 Publication	<1%

9	Enea Rezzonico, Sofiane Lariani, Caroline Barretto, Gabriella Cuanoud et al. "Global transcriptome analysis of the heat shock response of Bifidobacterium longum", FEMS Microbiology Letters, 2007 Publication	<1%
10	Aarif, Ovais, and Anjali Aggarwal. "Evaporative cooling in late-gestation Murrah buffaloes potentiates immunity around transition period and overcomes reproductive disorders", Theriogenology, 2015. Publication	<1%
11	Shoichi Maruyama. "Low circulating CD34+ cell count is associated with poor prognosis in chronic hemodialysis patients", Kidney International, 12/2008 Publication	<1%
12	Malik, Uzma, Aneela Javed, Amjad Ali, and Kashif Asghar. "Structural and functional annotation of human FAM26F: A multifaceted protein having a critical role in the immune system", Gene, 2016. Publication	<1%
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14	Raheel, Ummar, Muhsin Jamal, and Najam Us Sahar Sadaf Zaidi. "A Molecular Approach Designed to Limit the Replication of Mature DENV2 in Host Cells", Viral Immunology, 2015. Publication	<1%
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18	Huijun Wang, Zhengdong Zhang, Ruiyun Li, K. Kian Ang, Huazhong Zhang, Nancy P. Caraway, Ruth L. Katz, Feng Jiang. "Overexpression of S100A2 protein as a prognostic marker for patients with stage I non small cell lung cancer", International Journal of	<1%

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19 Zaira Rehman, Ammad Fahim, Attya Bhatti, Hajra Sadia, Peter John. "Co-expression of HIF-1α, MDR1 and LAPTM4B in peripheral blood of solid tumors", PeerJ, 2019 <1%
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