HHS Public Access

Author manuscript

Hum Pathol. Author manuscript; available in PMC 2015 May 12.

Published in final edited form as:

Hum Pathol. 2014 April ; 45(4): 701–708. doi:10.1016/j.humpath.2013.11.001.

Molecular Characterization of Gallbladder Cancer using Somatic Mutation Profiling

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Abstract

Gallbladder cancer is relatively uncommon with high incidence in certain geographic locations, including Latin America, East and South Asia and Eastern Europe. Molecular characterization of this disease has been limited and targeted therapy options for advanced disease remain an open area of investigation. In the present study, surgical pathology obtained from resected gallbladder cancer cases (n=72) was examined for the presence of targetable, somatic mutations. All cases were formalin-fixed and paraffin-embedded (FFPE). Two approaches were used: a) mass spectroscopy-based profiling for 159 point ('hot-spot') mutations in 33 genes commonly involved in solid tumors and b) next-generation sequencing (NGS) platform that examined the complete coding sequence of in 182 cancer-related genes. Fifty-seven cases were analyzed for hotspot mutations and 15 for NGS. Fourteen hotspot mutations were identified in nine cases. Of these, *KRAS* mutation was significantly associated with poor survival on multivariate analysis. Other targetable mutations included *PIK3CA* (N=2) and *ALK* (N=1). On NGS, 26 mutations were noted in 15 cases. *P53* and PI3 kinase pathway (*STK11, RICTOR,TSC2)* mutations were common. One case had FGF10 amplification while another had *FGF3-TACC* gene fusion, not previously described in gallbladder cancer. In conclusion, somatic mutation profiling using archival FFPE samples from gallbladder cancer is feasible. NGS, in particular may be a useful platform for identifying novel mutations for targeted therapy.

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Conflict of Interest: None

Keywords

gallbladder neoplasms; mutational analysis; DNA Sequencing

INTRODUCTION

Gallbladder cancer affects over 140,000 patients annually worldwide and over 100,000 will die each year from this disease.(1) Women are affected more than men and in the U.S.; Hispanic population and Alaskan natives have a disproportionately high incidence of gallbladder cancer.(2) There is a remarkable geographic variation with the highest incidence rates reported in India, Korea, Japan, Czech Republic, Slovakia, Spain, Columbia, Chile, Peru, Bolivia, and Ecuador. Etiologies include chronic cholelithiasias, *Salmonella* infections, toxin exposure, obesity and rarely due to genetic diseases like Hereditary Non-Polyposis Cancer Coli (HNPCC) and type 1 neurofibromatosis. Gallbladder cancer is thought to be at least partly the consequence of chronic inflammation-induced genetic changes.

The current molecular profiling data of gallbladder cancer are limited to small case series or case reports that include one or more oncogenes. High-throughput screening for targetable mutations in this disease is lacking. An understanding of the molecular characteristics and heterogeneity of gallbladder cancer is critical towards improving the treatment paradigm for this disease. An impetus for such characterization is the potential of targeted therapies directed against the products of these molecular aberrations including the tumor proteomic profile. Once the underlying molecular abnormalities of a cancer are identified, targeted inhibitors can be discovered and result in incremental benefit even in genetically heterogeneous malignancies. For instance, in lung cancer the identification of echinoderm microtubule associated protein like 4 - anaplastic lymphoma kinase (*EML4-ALK)* mutation has led to a targeted approach with crizotinib and tumors with epidermal growth factor receptor (*EGFR*) mutations to the development of erlotinib or gefitinib.(3) High-throughput technologies that can rapidly screen for somatic mutations in archival formalin-fixed, paraffin-embedded specimens are critical for this effort. The Sequenom Massarray™ system is ideally suited for the detection of low abundance mutations and can be customized towards targeted therapeutics.(4, 5) In the present study, we used the high-throughput Sequenom MassArray™ approach to investigate mutations in 33 genes in a cohort of gallbladder cancer cases to determine the frequency of genetic mutations in this population. We also explored next generation sequencing (NGS) to examine a wider panel of genetic aberrations in a limited number of gallbladder cancer cases.

MATERIAL AND METHODS

Tumor samples

Surgically resected, formalin fixed paraffin embedded (FFPE) specimens were obtained for 72 patients with gallbladder cancer. The paraffin embedded blocks were sectioned, and hematoxylin & eosin (H&E) stained slides were reviewed by surgical pathology to confirm the tumor content in each section. Ten serial sections (4μm) were cut from selected tissue

blocks and areas with tumor tissue were micro dissected from those slides using the H&E slides as templates. Approval for the study was obtained from the institutional review board at MD Anderson Cancer Center.

DNA Extraction

The samples were deparaffinized using xylene washes followed by ethanol (100%) washes. DNA extraction was performed using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer protocol. DNA was quantitated using the NanoQuant system (Tecan Group, Männedorf, Switzerland).

Sequenom MassArray

Hotspot mutational analysis was performed using the Sequenom MassARRAY™ using the iPLEX™ technology (Sequenom, Inc, San Diego, CA). This technology allows for parallel high-throughput screening while using minimal DNA obtained from FFPE specimens (6). Mutations were screened by using amplification through polymerase chain reaction (PCR) and single-base primer extension where the wild type or mutated base was identified by mass spectrometry. Briefly, for each mutation site, PCR and extension primers were designed using Sequenom, Inc. Assay Design. PCR reactions were run following manufacture's protocol. After PCR, amplicons were cleaned using EXO-SAP® kit (Sequenom) in a GeneAmp 9700 thermocycler (Applied Biosystems). Then the primer was then extended by IPLEX™ chemistry, desalted using Clean Resin (Sequenom), and spotted onto SpectroChip matrix chips (Sequenom) using a nanodispenser (Samsung). Chips were run in duplicate on a Sequenom MassArray Matrix-assisted laser desorption/ionization-Time of Flight (MALDI-TOF) MassArray system. We used Sequenom Typer Software for visual inspection and interpretation of mass spectra. Reactions where the mutant peak represented more than 10% of the wild type peak were scored as positive. The data analysis was performed using MassArray TYPER 4.0 genotyping software (Sequenom) where the SNP calls were divided in 3 groups: conservative, moderate and aggressive calls, depending on the level of confidence.

The Sequenom panel used here was previously designed by the Characterized Cell Line Core (Core Shared Resources – CCSG) at MD Anderson Cancer Center with the aim of detecting somatic DNA alterations in cancer samples. The Sequenom panel was designed based on data form the Catalogue of Somatic Mutations in Cancer (COSMIC) and the Cancer Genome Atlas (TCGA) that reported those alterations (and others in the panel) as somatic mutations previously. A total of 159 point mutations in 33 genes frequently mutated in solid tumors including were analyzed. The analytical sensitivity of the assay [limit of detection (LOD) 5%–10% of mutant DNA in total DNA] is higher than conventional Sanger sequencing (LOD: 10%–20%) and similar to pyrosequencing (LOD: 5%–10%). The advantages offered by the MassARRAY system include high-throughput screening for many hot-spot mutations in parallel, use of minimal DNA isolated from formalin-fixed paraffin-embedded tissues, ability to detect coexisting multiple mutations, and cost and time effectiveness. Appendix 1 lists the genes and mutations investigated in this study.

Next Generation Sequencing

The pathologic diagnosis of each case of gallbladder cancer was confirmed on routine hematoxylin- and eosin-stained slides. All samples sent for DNA extraction contained a minimum of 20% DNA derived from tumor cells. DNA was extracted from 40 mm of FFPE tissue using the Maxwell 16 FFPE Plus LEV DNA Purification kit (Promega™) and quantified using a standardized PicoGreen fluorescence assay (Invitrogen™). Library construction was performed as described previously, using 50–200 ng of DNA sheared by sonication to B100–400 bp before end-repair, dA addition and ligation of indexed, Illumina™ sequencing adaptors (7, 8). Enrichment of target sequences (3320 exons of 182 cancer-related genes and 37 introns from 14 genes recurrently rearranged in cancer representing approximately 1.1Mb of the human genome) was achieved by solution-based hybrid capture with a custom Agilent SureSelect™ biotinylated RNA baitset (8). The selected libraries were sequenced on an Illumina HiSeq 2000 platform using 49149 paired-end reads. Sequence data from genomic DNA was mapped to the reference human genome (hg19) using the Burrows-Wheeler Aligner™ and were processed using the publicly available Sequence Alignment/Map (SAMtools), Picard and Genome Analysis Toolkit (9, 10). Point mutations were identified by a Bayesian algorithm; short insertions and deletions determined by local assembly; gene copy number alterations (amplifications) by comparison to process matched normal controls; and gene fusions/rearrangements were detected by clustering chimeric reads mapped to targeted introns as described previously (11).

Statistical Analysis

Given the limited number of cases analyzed for NGS, only the cases analyzed for hotspot mutations (n=57) were analyzed for their association with survival. Overall survival (OS) was calculated as the number of months from surgery (or core biopsy) to death or last follow-up date. Patients who were alive at their last follow-up were censored on that date. Time to Progression (TTP) was calculated as the number of months from surgery (or core biopsy) to progression. Patients without tumor progression at their last follow-up were censored on that date. The Kaplan-Meier product limit method was used to estimate the median OS for each clinical/demographic factor.(12) Univariate Cox proportional hazards regression was used to model the association between potential predictors and OS. Multivariate Cox proportional hazards regression was used to model all the statistically significant variables in the univariate setting. Backwards selection method was used to remove variables that did not remain significant in the multivariate model.(13) For each factor, medians, hazard ratios (HR), their 95% confidence intervals (CI), and proportional hazards regression p-values are presented in tables. Similar analyses were performed for time to progression. Statistical significance was considered at P-values of <0.05.

Statistical analysis was performed using STATA/SE version 12.1 statistical software (Stata Corp. LP, College Station, TX).

RESULTS

Fifty-seven cases of gallbladder cancer were analyzed for hotspot mutations and 15 for NGS. Patient demographics are described in Table 1. Fourteen hotspot mutations (Table 2)

were identified from eleven different tumors within this sample set, with three cases demonstrating more than 1 mutation. *IDH1* mutations were the most frequent (n=4). The others identified included mutations of *KRAS* (n=3), *NRAS* (n=3), *PIK3CA* (n=2) and *MET* (n=1). Of these, *IDH1* and *MET* may represent germline polymorphisms rather than somatic mutations as discussed below. Figure 1 demonstrates the *PIK3CA, IDH1 and KRAS* mutations. Figures 2A–2D depict the histologies (H&E) of four gallbladder cancer cases along with their corresponding mutations. A total of 36/57 (63.2%) patients enrolled in the study have expired to date. A univariate survival analysis on these data demonstrated a significant relationship of overall survival with six factors. The overall risk of mortality was associated with treatment with chemotherapy (HR: 2.84; 95%CI: 1.23–6.53; p=0.014), lymphatic infiltration (HR: 2.72; 95%CI: 1.22–6.04; p=0.014), venous infiltration (HR: 2.27; 95%CI: 1.08–4.79; p=0.031), perineural infiltration (HR: 2.14; 95%CI: 1.06–4.33; p=0.033), positive *KRAS* mutation (HR: 3.56; 95%CI: 1.06–11.92; p=0.040), and with a positive *IDH1* mutation (HR: 4.04; 95%CI: 1.35–12.13; p=0.013) (Fig.3a). In addition, patients who had chemotherapy were at greater risk of progressing than non-treated patients (HR: 13.82; 95%CI: 1.84–103.84; p=0.011).

A multivariate analysis of overall survival was also performed using backward elimination methods. Overall survival was seen to be associated with patients age 62–79 (HR: 5.93; 95%CI: 1.76 – 20.00; p=0.004), and age ≥ 70 (HR: 3.84; 95%CI: 1.19 – 12.39; p=0.024), clinical stages 3a, 3b, 4a & 4b (HR: 2.60; 95%CI: 1.03–6.59; p=0.044), venous infiltration (HR: 3.42; 95%CI: 1.46–8.03; p=0.005) and *KRAS* (HR: 8.91; 95%CI: 1.99–39.94; p=0.004-Fig.3b).

On NGS, 26 mutations were noted in 15 cases (Tables 3). *P53* was most common and there was relative preponderance of mutations involving the PI3 kinase pathway: *STK11, RICTOR, TSC2*. Two cases had FGF pathway aberrations: FGF10 amplification and one case of *FGF3-TACC* fusion gene (Fig 4). Two cases are illustrated wherein the mutational data were utilized for targeted therapeutics with success (Fig 5a; Fig 5b).

DISCUSSION

Gallbladder cancer has been referred to as an ‘orphan’ cancer, given its relative infrequency in the Western population. Molecular research in this disease has lagged behind the commoner gastrointestinal cancers, such as colorectal and gastric cancer. The known genetic alterations include mutations of *K-RAS* (in 3–40%, more likely in East Asia), *PI3KCA* (12%), *p53* (40%) and *BRAF* (33%) oncogenes, and amplification of *Her-2/ Neu* (15%).(14, 15) Other genetic alterations described include loss of expression fragile histidine triad (*FHIT*) gene, microsatellite instability, overexpression of P13-K/Akt, VEGF and p21.(16, 17) Key limitations of the above data include the small number of cases tested, geographic and ethnic variation.

The present study, to our knowledge represents the largest number of surgically resected gallbladder cancer cases that had somatic mutation profiling. All of our specimens were FFPE and therefore we chose a platform that had non-fastidious DNA requirements and could detect low-abundance mutations. Sequenom Massarray technique is ideal in this

situation for profiling single nucleotide mutations and polymorphisms. A limitation of this retrospective study is that we did not have parallel blood or normal tissue to assess if the mutations we noted were germline or somatic. We have used preselected panels, which included targetable oncogenes from the COSMIC and TCGA database. While the plan was to include somatic mutations only, in these panels, subsequent studies have reported that at least two of the genetic alterations (*IDH1* and *met*) were germline.

Sanger sequencing has been effectively used for somatic mutation discovery. However, when there is a heterogeneous mixture of cancerous and normal tissue, Sanger sequencing may be unable to detect low frequency mutations. In one published study, sequencing failed to detect *EGFR* (Epidermal Growth Factor Receptor) mutations in tumors with roughly 10% allele frequencies.(18) Clinical somatic mutation detection will require high degree of sensitivity than standard sequencing. The Massarray™ system combines PCR with matrix-assisted laser desorption/ ionization time of flight mass spectrometry for rapidly multiplexed nucleic acid analysis. Furthermore, this system can rapidly profile hundreds of mutations in FFPE samples with as little as 5% mutation abundance with a short turn-around time. However, the podisadvantages of this approach is that these multiplex genomic tests only detect the expression of pre-selected hotspot mutations and do not lead to the discovery of novel targets. This limitation is particularly relevant to the less common tumors, such as gallbladder cancer.

Our findings indicated that *IDH1_V178I* was the commonest DNA variation on Sequenom Massarray. It is estimated that another mutation on *IDH1_R132* occurs in upto 20% of high grade glioma and this mutation is associated with a better prognosis and response to therapy. (19) On the other hand, the same somatic mutation in acute myeloid leukemia is associated with a poor prognosis and lack of complete response, particularly in otherwise cytogenetically normal cases.(20) A poor prognosis was noted in our study with *IDH1_V178I* mutation. In a prior study, *IDH1* mutations (*IDH1_R132)* were noted in cholangiocarcinoma, but none were noted in the 25 cases of gallbladder cancer studied. (21) Isocitrate dehydrogenase (IDH) catalyzes the conversion of isocitrate to α-ketoglutarate and mutations in this pathway is a relevant target for therapy given the development of IDH inhibitors.(22) These mutations also conferred an enzymatic gain-of-function: the novel NADPH-dependent reduction of α-ketoglutarate to the normally trace metabolite *R*(−)-2-hydroxyglutarate (2-HG), which is oncogenic.(23) Measurement of intracellular 2-HG can therefore be used to assess the functional impact of the mutation. In case of *IDH1_V178I*, no elevation of 2-HG was noted, which raises the question of whether this mutation represents a non-functional polymorphism or if the functional oncogenic effect includes a non 2-HG metabolic pathway. Several SNPs related to the lipid metabolism, estrogen receptor and DNA repair have been associated with survival in gallbladder cancer (24–26). One case had *ALK* mutation (*ALK_F1174L_C3522AG*), which has not yet been described in this disease and offers effective targeted therapy options.

The next generation sequencing approach offers several advantages over the traditional methods, including the ability to simultaneously sequence hundreds of genes in a single test, have a higher depth of coverage and thereby heightened sensitivity for mutation detection, ideal for ‘precision medicine’.(27) In addition, these technologies can detect deletions,

amplifications, translocations and base substitutions at a relatively rapid rate. The disadvantage includes cost, high computational requirements and high tissue requirement that make the technology unsuitable for smaller biopsies, circulating tumor cells and circulating plasma DNA. A notable finding in our study was the relatively common occurrence PI3-kinase pathway mutations (*TCS2, STK11, RICTOR*), which opens potential options for targeted therapies directed against these proteins. Deshpande *et al*, had noted the relative frequency of *PI3KCA* mutations in this population.(21) Other targetable mutations included *AURKA and BAP*, which may potentially be treated with aurora kinase inhibitors and DNA damaging agents [such as cisplatin and poly ADP ribose polymerase (PARP) inhibitors], respectively.

A novel finding in our study was the detection of fusion between Fibroblast Growth Factor Receptor (*FGFR3)* and Transforming Acidic Coiled-Coil *(TACC)* [in-frame fusion between exons 1–17 of *FGFR3* (containing the kinase domain) and exons 11 to the C-terminus of *TACC3* (containing the coiled coil TACC domain)]. The FGFR family plays an important role in cellular proliferation and angiogenesis and gain of function mutations in FGFRs have been reported in several malignancies.(28) *FGFR3* mutation or amplification has not been reported in gallbladder cancer to our knowledge. Similar fusions between *FGFR3* and *TACC3* have recently been reported in a small percentage of glioblastomas.(29) These fusions have also recently been described in cholangiocarcinoma, are proven to be oncogenic and the resulting tumors may be susceptible to FGFR inhibitors.(30)

In conclusion, gallbladder cancer is amenable to precise interventions with targeted therapies and novel sequencing techniques may provide prognostic and therapeutic opportunities.

Acknowledgments

Funding Disclosures: Supported by a grant from Global Academic Programs, MD Anderson Cancer Center and Project Fondecyt 1120208.

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Appendix 1

Appendix 1

GENES AND MUTATIONS INVESTIGATED

AKT1_E17K_G49A	FGFR1_S125L_C374T	MET_Y1248C_A3743G
AKT2_E17K_G49A	FGFR2_N549KK_T1647GA	MET_Y1248HD_T3742CG
AKT3_E17K_G49A	FGFR2_S252W_C755G	MET_Y1253D_T3757G
ALK_F1174CS_T3521GC	FGFR3_G370C_G1108T	MGA_T1747N_C5421A
ALK_F1174L_C3522AG	FGFR3_G380R_G1138A	NRAS_A146T_G436A
ALK_F1174LIV_T3520CAG	FGFR3_G697C_G2089T	NRAS_G12DAV_G35ACT
ALK_F1245C_T3734G	FGFR3_K650MT_A1949TC	NRAS_G12SRC_G34ACT
ALK_F1245L_C3735AG	FGFR3_R248C_C742T	NRAS_G13DAV_G38ACT
ALK_F1245VI_T3733GA	FGFR3_S249C_C746G	NRAS_G13SRC_G37ACT
ALK_I1171N_T3512A	FGFR3_Y373C_A1118G	NRAS_Q61EKX_C181GAT

ALK_R1275QL_G3824AT	FOXL2_C134W_C402G	NRAS_Q61HHQ_A183TCG
BCOR_N1407STI_A4220GCT	GNA11_Q209LP_A626TC	NRAS_Q61RPL_A182GCT
BRAF_D594GV_A1781GT	GNA11_R183C_C547T	PDGFRA_D842V_A2525T
BRAF_E586K_G1756A	GNAQ_Q209H_A627T	PDGFRA_D842YN_G2524TA
BRAF_G464EVA_G1391ATC	GNAQ_Q209LPR_A626TCG	PDGFRA_N659K_C1977A
BRAF_G466EVA_G1397ATC	GNAS_R201H_G602A	PDGFRA_N659Y_A1975T
BRAF_G466R_G1396CA	GNAS_R201SC_C601AT	PDGFRA_V561D_T1682A
BRAF_G469EVA_G1406ATC	GRM3_E870K_G2608A	PIK3CA_A1046V_C3137T
BRAF_G469R_G1405CA	IDH1_G70D_G209A	PIK3CA_C420R_T1258C
BRAF_K601E_A1801G	IDH1_R132CGS_C394TGA	PIK3CA_E110K_G328A
BRAF_L597RQ_T1790GA	IDH1_R132HL_G395AT	PIK3CA_E418K_G1252A
BRAF_V600_G1800	IDH1_V178I_G532A	PIK3CA_E453K_G1357A
BRAF_V600EAG_T1799ACG_F	IDH2_R140LQ_G419TA	PIK3CA_E542KQ_G1624AC
BRAF_V600EAG_T1799ACG_R	IDH2_R140W_C418T	PIK3CA_E542VG_A1625TG
BRAF_V600LM_G1798TA	IDH2_R172GW_A514GT	PIK3CA_E545AGV_A1634CGT
CC2D1A_L913V_C3036G	IDH2_R172MK_G515TA	PIK3CA_E545D_G1635CT
CDK4_R24C_C70T	IDH2_R172S_G516T	PIK3CA_E545KQ_G1633AC
CDK4_R24H_G71A	JAK2_V617F_G1849T	PIK3CA_F909L_C2727G
CSMD1_A409S_G1225T	KIT_D816GVA_A2447GTC	PIK3CA_G118D_G353A
CSMD1_Q3005X_C9013T	KIT_D816HNY_G2446CAT	PIK3CA_H1047RL_A3140GT_F
CTNNB1_D32AGV_A95CGT	KIT_K642E_A1924G	PIK3CA_H1047RL_A3140GT_R
CTNNB1_D32HNY_G94CAT	KIT_L576P_T1727C	PIK3CA_H1047Y_C3139T
CTNNB1_G34EVA_G101ATC	KIT_N566D_A1696G	PIK3CA_H701P_A2102C
CTNNB1_H36PRY_A107CGT	KIT_N822KNK_T2466GCA	PIK3CA_K111N_G333C
CTNNB1_I35NST_T104AGC	KIT_N822YHD_A2464TCG	PIK3CA_M1043I_G3129ATC
CTNNB1_S33APT_T97GCA	KIT_R634W_C1900T	PIK3CA_M1043V_A3127G
CTNNB1_S37CFY_C110GTA	KIT_V559ADG_T1676CAG	PIK3CA_N345K_T1035A
CTNNB1_S45APT_T133GCA	KIT_V560DGA_T1679AGC	PIK3CA_P539R_C1616G
CTNNB1_S45CFY_C134GTA	KIT_V825A_T2474C	PIK3CA_Q060K_C178A
CTNNB1_T41APS_A121GCT	KIT_Y553N_T1657A	PIK3CA_Q546EK_C1636GA
CTNNB1_T41INS_C122TAG	KRAS_A146PT_G436CA	PIK3CA_Q546LPR_A1637TCG
EGFR_G719CS_G2155TA	KRAS_G10R_G28A	PIK3CA_R088Q_G263A
EGFR_K860I_A2579T	KRAS_G12DAV_G35ACT	PIK3CA_S405F_C1214T
EGFR_L858R_T2573G	KRAS_G12SRC_G34ACT	PIK3CA_T1025SA_A3073TG
EGFR_L861QR_T2582AG	KRAS_G13DAV_G38ACT	PIK3CA_Y1021C_A3062G
EGFR_S720P_T2158C	KRAS_G13SRC_G37ACT	PIK3CA_Y1021HN_T3061CA
EGFR_T790M_C2369T	KRAS_Q61EKX_C181GAT	PPP2R1A_W257G_T769G
EGFR_T854I_C2561T	KRAS_Q61HHQ_A183CTG	RAF1_A319S_G955T
EGFR_Y813C_A2438G	KRAS_Q61LPR_A182TCG	RAF1_L613V_C1837G
EPHA3_K761NN_G2283TC	MAP2K2_E207KQ_G619AC	RAF1_N115S_A344G

FBXO4_L23Q_T68A	MAP2K7_D290D_C870T	RAF1_Q335H_G1005C
FBXO4_P76T_C226A	MAP2K7_R162H_G485A	RAF1_S259A_T775G
FBXO4_S8R_C24AG	MAP2K7_S271T_T811A	RAF1_Y340D_T1018G
FBXW7_R465C_C1393T	MAP2K7_S311L_C932T	RET_M918T_T2753C
FBXW7_R465HL_G1394AT	MET_H1112RL_A3335GT	RPL22_K15TRM_A44CGT
FBXW7_R479QL_G1436AT	MET_H1112Y_C3334T	SFRS9_Y192X_C722A
FBXW7_R505CS_C1513TA	MET_M1268T_T3803C	SMO_A324T_G970A
FBXW7_R505HLP_G1514ATC	MET_N375S_A1124G	SRC_Q531X_C1591T
FBXW7_S582L_C1745T	MET_R988C_C2962T	TGM2_S212P_T734C
FGFR1_P252T_C754A	MET_T1010I_C3029T	

Appendix 2

APPENDIX 2

GENETIC MUTATIONS SEQUENCED USING NGS

182 genes sequenced across entire coding sequence				
Gene	**Gene**	**Gene**	**Gene**	**Gene**
ABL1	CDK6	FLT4	MEN1	PTPN11
ABL2	CDK8	FOXP4	MET	PTPRD
AKT1	CDKN2A	GATA1	MITF	RAF1
AKT2	CDKN2B	GNA11	MLH1	RARA
AKT3	CDKN2C	GNAQ	MLL	RB1
ALK	CEBPA	GNAS	MPL	RET
APC	CHEK1	GPR124	MRE11A	RICTOR
AR	CHEK2	GUCY1A2	MSH2	RPTOR
ARAF	CRKL	HQXA3	MSH6	RUNX1
ARFRP1	CRLF2	HRAS	MTOR	SMAD2
ARID1A	CTNNB1	HSP9OAA1	MUTYI-1	SMAD3
ATM	DDR2	IDH1	MYC	SMAD4
ATR	DNMT3A	IDH2	MYCL1	SMARCA4
AURKA	DOT1L	IGF1R	MYCN	SMARCB1
AURKS	EGFR	IGF2R	NFl	SMO
BAP1	EPI-1A3	IKBKE	NF2	SOX1O
BCL2	EPF-1A5	IKZF1	NKX2-1	SOX2
BCL2A1	EPHA6	INHBA	NOTCH1	SRC
BCL2L1	EPHA7	INSR	NPM1	STAT3
BCL2L2	EPHB1	IRS2	NRAS	STK11
BCL6	EPHB4	JAK1	NTRK1	SUFU
BRAF	EPHB6	JAK2	NTRK2	T5X22
BRCA1	ERBB2	JAK3	NTRK3	TET2

BRCA2	ERBB3	JUN	PAK3	TGFBR2
CARD11	ERBB4	KDM6A	PAX5	TNFAIP3
CBL	ERCC2	KDR	PDGFRA	TNKS
CCND1	ERG	KIT	PDGFRB	TNKS2
CCND2	ESR1	KRAS	PHLPP2	TOP1
CCND3	EZH2	LRP1B	PIK3CA	TP53
CCNE1	FANCA	LRP6	PIK3CG	TSC1
CD79A	FBXW7	LTK	PIK3R1	TSC2
CD79B	FGFR1	MAP2K1	PKHD1	USP9X
CDH1	FGFR2	MAP2K2	PLCG1	VHL
CDH2	FGFR3	MAP2K4	PRKDC	WT1
CDH2O	FGFR4	MCL1	PTCH1	
CDH5	FLT1	MDM2	PTCH2	
CDK4	FLT3	MDM4	PTEN	
14 genes sequenced across selected iritrons				
Gene				
ALK				
BCR				
BRAF				
EGFR				
ETV1				
ETV4				
ETV5				
ETV6				
EWSR1				
MLL				
RAF1				
RARA				
RET				
TMPRSS2				

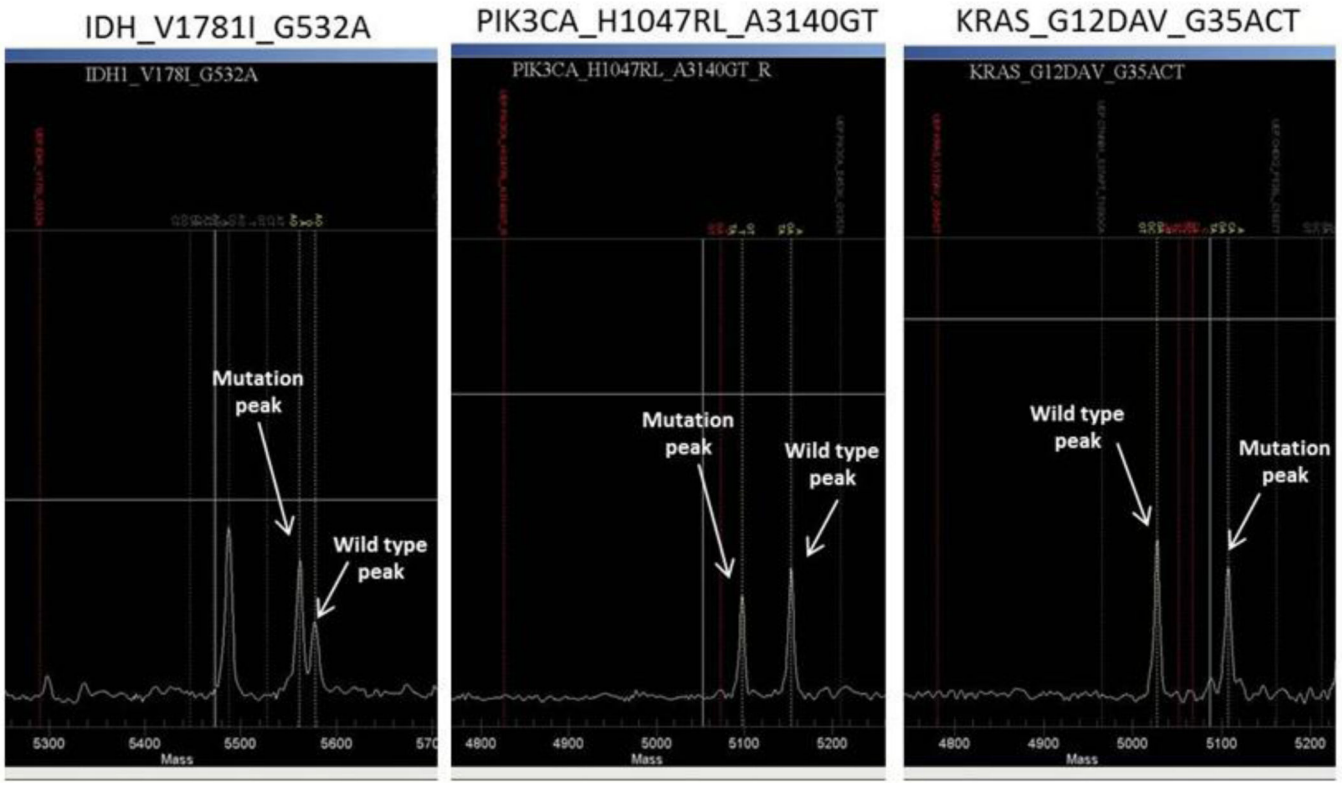

Figure 1.
Peaks for PIK3CA, IDH1 and KRAS mutations (Sequenom Massarray)

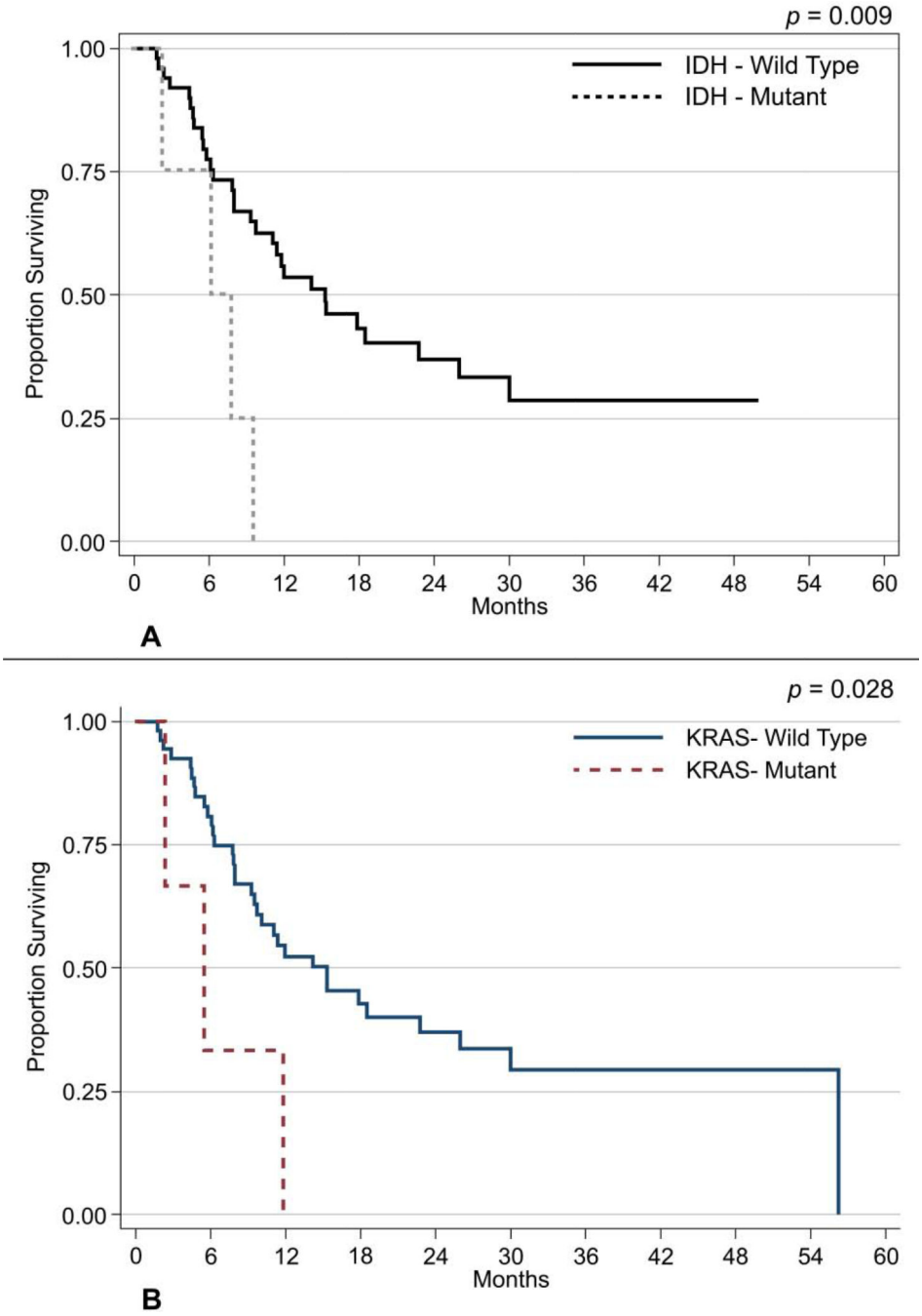

Figure 2.

2a) IDH1 mutation and association with overall survival.

2b) KRAS mutation and association with overall survival

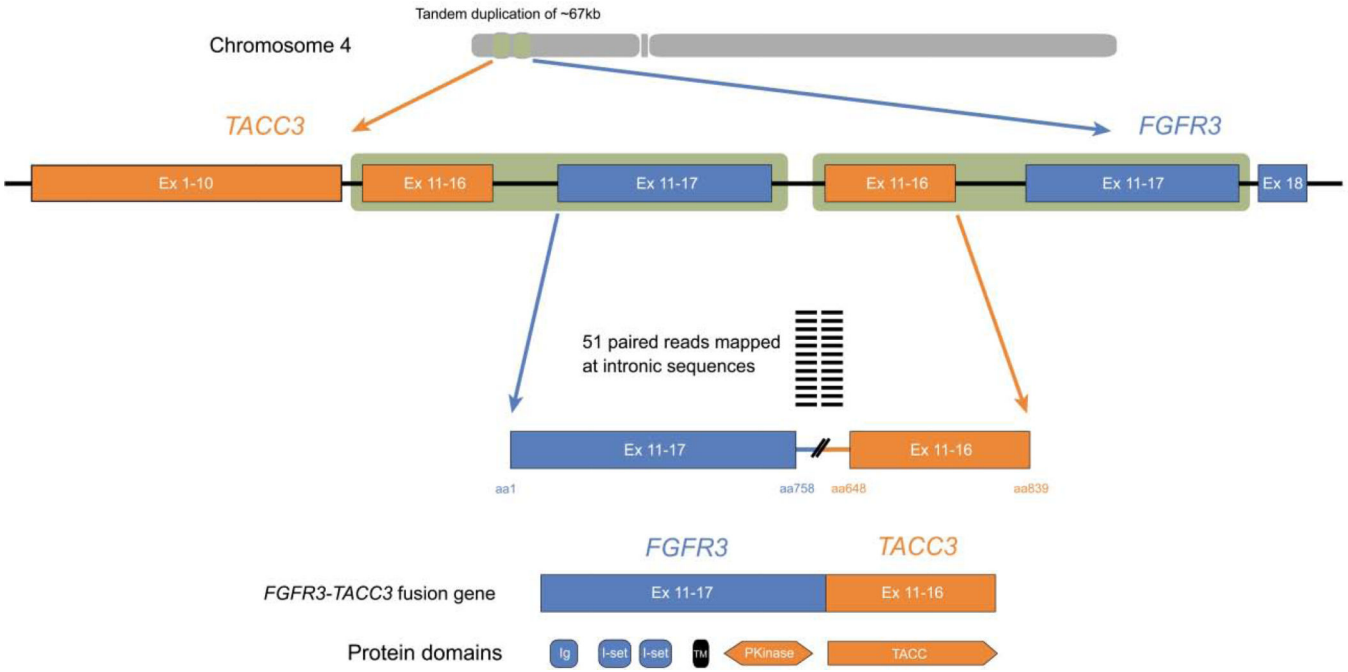

Figure 3.
Schematic of FGFR3-TACC3 Fusion Gene in Gallbladder Cancer

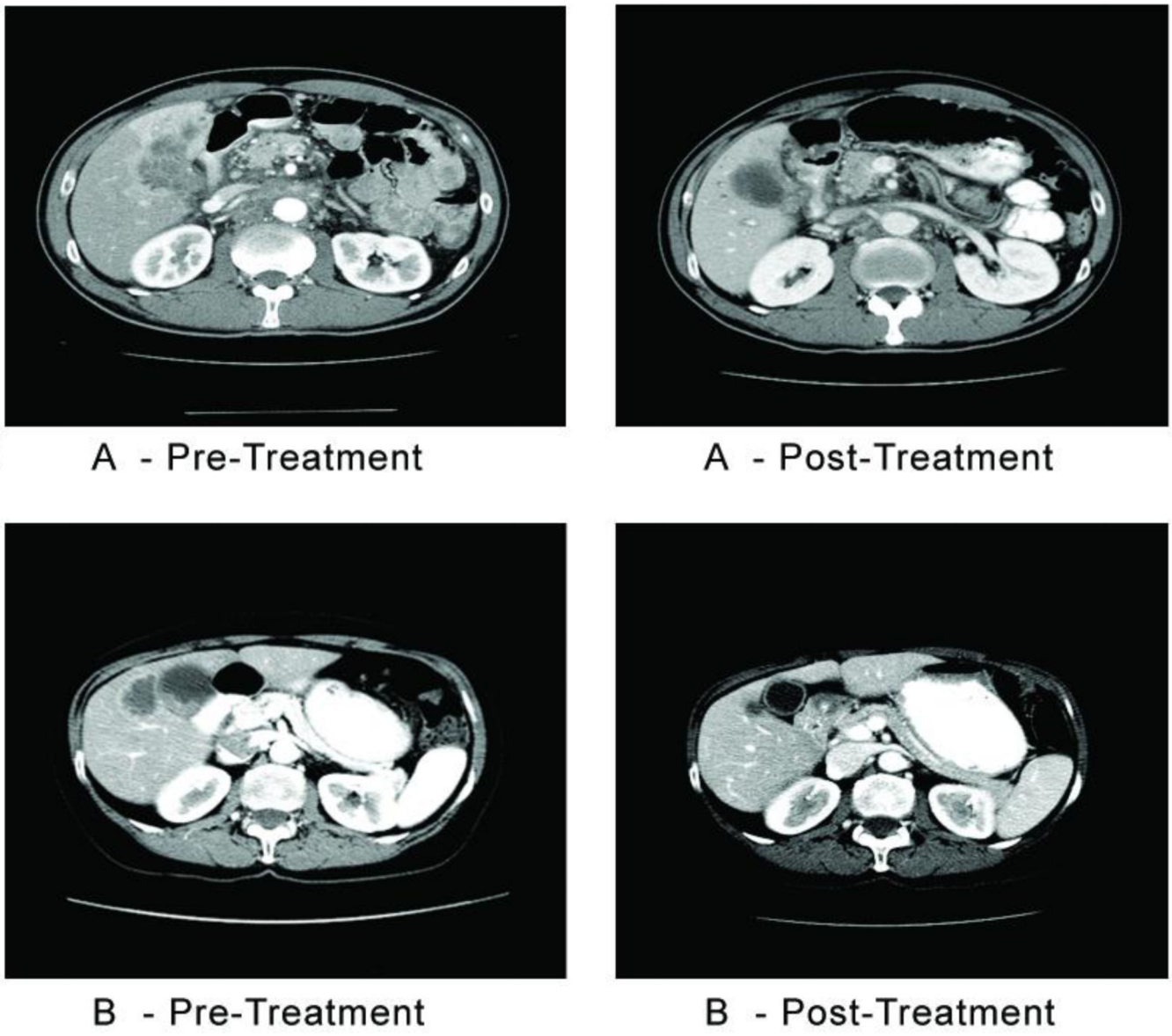

Figure 4.

Illustrations of mutational data successfully utilized for targeted therapeutics.

4a) Erlotinib in combination with gemcitabine and oxaliplatin before therapy and 4 months post therapy.

4b) Trastuzumab in combination with 5-fluorouracil, leucovorin and oxaliplatin as second-line therapy before therapy and 3 months post-therapy.

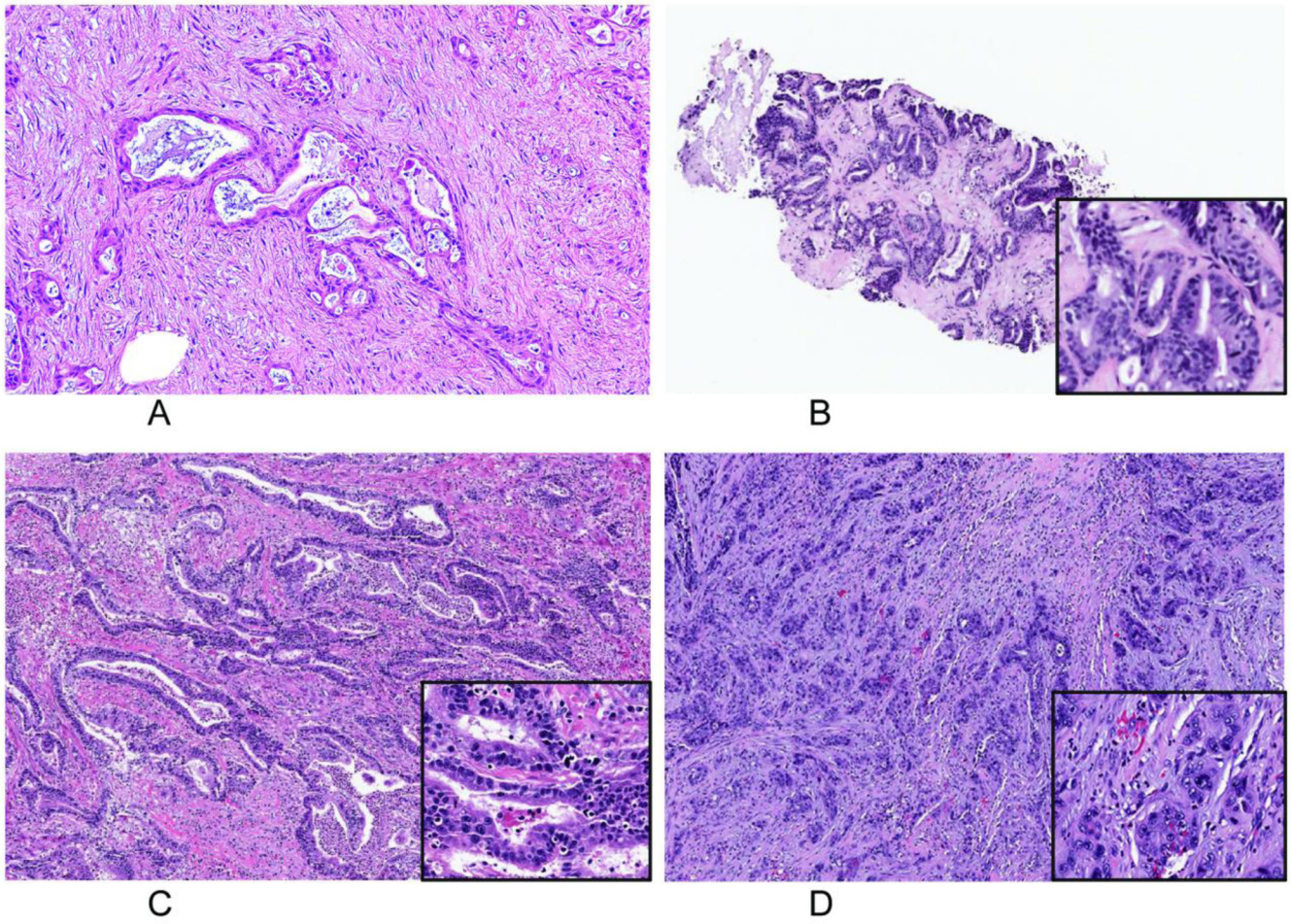

Figure 5.

Representative histopathology of samples with corresponding mutations used for Sequenom analysis and NGS.

5A) KRAS

5B) TP53, ERBB2

5C) FGFR3-TACC3, CCNE1, MCL1, MYC, TP53

5D)ARID1A

Table 1

Summary Statistics of Patient Demographics and Tumor Characteristics

CHARACTERISTICS	Type of Analysis	
	Hotspot Mutation Analysis (N = 57)	**Next Generation Sequencing (N=15)**
Age (years)	**MEDIAN (RANGE) 62 (30–84)**	**MEDIAN (RANGE) 62 (48–78)**
	N (%)	**N (%)**
Sex		
Male	25 (44%)	5 (33%)
Female	32 (56%)	10 (67%)
Ethnicity		
Asian	1 (2%)	1 (7%)
Hispanic	8 (14%)	1 (7%)
Black	5 (9%)	0 (0%)
White	43 (75%)	13 (86%)
Type of Surgery		
None	3 (5%)	4 (27%)
Simple (Laparoscopic)	31 (54%)	6 (40%)
Radical	23 (41%)	5 (33%)
Adjuvant Therapy		
Chemotherapy	25 (44%)	13 (87%)
Chemotherapy & Radiation	11 (19%)	2 (13%)
None	21 (37%)	0 (0%)
Histological Type		
Adenocarcinoma	51 (90%)	15 (100%)
Adenosquamous	4 (7%)	0 (0%)
Carcinosarcoma	2(3%)	0 (0%)
Degree of Differentiation		
Poor	16 (28%)	7 (47%)
Moderate	38 (67%)	6 (40%)
Well	2 (4%)	2 (13%)
N/A	1 (2%)	0 (0%)
Lymphatic Infiltration*		
No	21 (39%)	1 (10%)
Yes	33 (61%)	9 (90%)
Venous Infiltration*		

CHARACTERISTICS	Type of Analysis	
	Hotspot Mutation Analysis (N = 57)	Next Generation Sequencing (N=15)
Age (years)	**MEDIAN (RANGE) 62 (30–84)**	**MEDIAN (RANGE) 62 (48–78)**
	N (%)	**N (%)**
No	22 (41%)	1 (10%)
Yes	32 (59%)	9 (90%)
Perineural Infiltration*		
No	29 (54%)	3 (30%)
Yes	25 (46%)	7 (70%)

N=Patient numbers;

*Surgical samples only (Hotspot N=54, NGS N=10)

Table 2

Genetic Mutations identified through Hotspot Analysis

Sample ID	Histology	Mutations
13	Adenocarcinoma	IDH1_V178I_G532A* PIK3CA_H1047RL_A3140GT
26	Adenosquamous	KRAS_G12DAV_G35ACT NRAS_Q61RPL_A182GCT
32	Adenocarcinoma	IDH1_V178I_G532A*
34	Adenocarcinoma	NRAS_G12DAV_G35ACT
42	Adenocarcinoma	IDH1_V178I_G532A*
44	Adenocarcinoma	KRAS_G12DAV_G35ACT MET_N375S_A1124G*
46	Adenocarcinoma	KRAS_G13DAV_G38ACT
47	Adenocarcinoma	IDH1_V178I_G532A*
49	Adenocarcinoma	PIK3CA_M1043I_G3129ATC
56	Adenocarcinoma	ALK_F1174L_C3522AG
57	Adenocarcinoma	NRAS_G12DAV_G35ACT

* Most likely to represent genomic variation (SNP)

Table 3

Genetic Alterations Identified Through NGS (N=15)

GENE	Alterations (With allele frequency or copy number)
TP53	V274F (10%) R282G (50%) R213* (29%) Y220C (2%) R342* (24%) C141* (21%) Splice site 559+1G>T (21%) F109V (46%) V272L
STK11	R86* (11%) E120* (15%) K62fs*98 (6%)
CCNE1	Amplification (copy no 11×) Amplification (copy no 13×)
MDM2	Amplification (copy no 6×) Amplification (copy no 16×)
MYC	Amplification (copy no 12×) Amplification (copy no 7×)
RICTOR	Amplification (copy no 12×) Amplification (copy no 7×)
APC	S2113fs*25 (21%)
ARID1A	G284fs*78 (18%)
AURKA	S398L (48%)
CDKN2A	Truncation - exon 1
CDKN2A/B	Loss Loss
CRKL	Amplification (copy no 12×)
FGF10	Amplification (copy no 7×)
FGFR3-TACC	FGFR3-TACC3 fusion, Amplification (copy no 8×)
KRAS	G12C, 3%
MCL1	Amplification (copy no 8×) Amplification (copy no 8×)
PRKAR1A	R97* (33%)
SMAD4	Truncation
SMARCA4	D558fs*6 (26%)
TSC2	Loss
BAP1	splice site 438-1delGTTTTTCCCC AG, 10% 1delGTTTTTCCCC AG, 10%
ERBB2	Amplification (copy no 20×) Amplification (copy no 9×)
PIK3CA	Amplification (copy no 7×)
ZNF703	Amplification (copy no 7×)