Circulating tumor DNA as prognostic marker of tumor recurrence/progression during the follow-up of patients on the waiting list of liver transplantation for hepatocellular carcinoma: a multicenter prospective study

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APPORT DE L'ADN TUMORAL CIRCULANT COMME MARQUEUR PRONOSTIQUE POST-TRANSPLANTATION HEPATIQUE POUR CARCINOME HEPATOCELLULAIRE : UNE ETUDE PROSPECTIVE MULTICENTRIQUE

THÈSE
PRÉSENTÉE POUR L'OBTENTION DU TITRE DE DOCTEUR EN MÉDECINE

DIPLÔME D'ÉTAT

Olivier MSIKA

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SOMMAIRE

1. PREAMBULE

Mon sujet de thèse a été choisi en concertation avec le Pr Thomas Decaens en 2017. J'étais alors naïf de toute connaissance sur le carcinome hépatocellulaire et en recherche scientifique. Je tenais absolument à mettre un pied dans le laboratoire et dans le monde de la recherche scientifique, ce pourquoi je lui ai demandé d'associer mon projet de thèse à un Master 2 de sciences. Ayant un attrait plus important pour l'oncologie, je lui ai demandé un sujet portant sur le carcinome hépatocellulaire. Après lui en avoir parlé, il m'a demandé de trouver un sujet qui m'intéressait et de lui présenter mes idées. Un mois plus tard, il m'a finalement dit qu'il avait « quelque chose pour moi ». Ma thèse portera sur l'ADN tumoral circulant dans le domaine du carcinome hépatocellulaire, sujet très en vogue dans le milieu de l'oncologie. Ce sujet m'a beaucoup plu.

Le Pr Decaens, m'a donc orienté vers un laboratoire d'excellence qui mène des projets de grandes envergures sur la génétique du carcinome hépatocellulaire, et qui est dirigé par le Pr Jessica Zucman-Rossi. Je suis donc revenu vivre près d'un an chez mes parents en région parisienne pour travailler dans ce laboratoire en 2019. Mon retour à Paris n'était pas évident, moi qui m'étais acclimaté au rythme paisible de la vie Grenobloise : redécouverte du métro, de la population, de la pollution et de la pluie, mais aussi des activités culturelles et sociales très enrichissantes.

Au laboratoire, j'ai pu découvrir un monde tout à fait différent de celui de l'hôpital, ou le mot « stress » n'existe pas et où la sérénité règne. J'ai donc effectué un premier travail pour récupérer les échantillons de la cohorte EFAPRE dont le Pr Decaens est l'investigateur principal et qui se trouvaient à l'hôpital Henri Mondor à Créteil. Après cette première étape j'ai pu apprendre le travail de « paillasse » grâce à l'aide précieuse des ingénieurs du laboratoire notamment Gabrielle Couchy, Bénédicte Noblet et Clément Meiller. Je leur dois beaucoup, car sans eux je n'aurais jamais pu mener mon projet à bout. Une fois avoir effectué toutes mes manipulations, je me suis rapproché des « experts » le Pr Zucaman-Rossi et le Dr Jean Charles Nault qui m'ont aussi beaucoup apporté quant au raisonnement scientifique. Je les remercie. Mon projet de thèse ne s'est fini qu'après avoir poursuivi les analyse bio-informatiques avec Sandrine Imbeaud qui a été d'une aide précieuse et qui m'a permis de finaliser ce projet à temps.

Je souhaite que cet article-thèse reflète mon travail et l'expérience acquise pendant cette période

2. INTRODUCTION FRANCAISE A L'ARTICLE

Le carcinome hépatocellulaire (CHC) est la première tumeur primitive maligne du foie. Dans plus de 80% des cas le CHC se développe dans un contexte de maladie chronique du foie ou cirrhose qui est en lien avec une infection chronique virale B (VHB), virale C (VHC), la consommation chronique d'alcool ou bien la NASH (Non-Alcoholic Steato Hepatitis). En 2030, les projections de l'OMS (Organisation Mondiale de la Santé), estiment que près d'un million de personnes décèderont du carcinome hépatocellulaire (1). En effet cette tumeur présente un pronostic péjoratif avec une survie à 5 ans inférieure à 20% ; et représente la seconde cause de mortalité liée à un cancer dans le monde après l'adénocarcinome du pancréas (2). Des programmes de dépistage chez les patients cirrhotiques existent en France et permettent à ces patients d'accéder à des thérapeutiques curatives. La mise en œuvre de programmes de vaccination contre l'hépatite B et le traitement de l'hépatite C ont permis de diminuer l'incidence du CHC lié aux hépatites virales dans les pays occidentaux. Cependant le virus de l'hépatite B reste la première cause de CHC dans le monde. L'augmentation de l'incidence du syndrome métabolique et de l'obésité a pour conséquence l'augmentation de cirrhose dite « NASH » ainsi que le risque de CHC (3).

La majorité des patients sont à ce jour diagnostiqués à un stade où un traitement curatif n'est malheureusement plus envisageable. Aujourd'hui et depuis 1999, la stratégie thérapeutique du CHC est dictée par la classification BCLC (Barcelona Clinic Liver Cancer) (4). Cette classification a permis de définir les stratégies thérapeutiques, selon le volume tumoral, la fonction hépatique et l'indice de performance des patients. Seuls les patients classés BCLC 0 ou A sont éligibles à un traitement à visée curative tels que l'ablation, la résection ou la transplantation hépatique.

La transplantation hépatique est le traitement idéal du CHC, puisqu'il permet une hépatectomie totale carcinologique et le traitement de l'hépatopathie sous-jacente qui reste un facteur de risque de développement de tumeur métachrone. Actuellement, en France, le CHC est la première indication de transplantation hépatique, et représente près de 30% des transplantations contre 13% au début des années 2000. Elle permet une survie à 5 ans comprise entre 63 et 80% dans cette indication, mais seulement 3 à 4% des patients atteints de CHC accèdent à la greffe. En France les critères d'attributions des greffons sont déterminés par le score AFP « alpha-fœtoprotéine » adopté par l'Agence de la Biomédecine en 2013 (5). Ce score a remplacé l'historique score de Milan (6) qui était considéré comme trop restrictif en excluant de nombreux patients de la liste de transplantation. Le score AFP est basé sur la taille de la plus grosse tumeur, le nombre de nodule(s) et le taux d'alpha-fœtoprotéine. Il est réévalué tous les trois mois, et autorise l'accès à la greffe s'il reste inférieur ou égal à 2. Cette limite a été fixée dans l'objectif d'isoler les patients à haut risque ou à faible risque de récidive post-transplantation hépatique.

En effet, compte tenu de la pénurie actuelle d'organe, le nombre de candidat pour un greffon hépatique était évalué à 2,4 en 2018 (7), il semble fondamental de sélectionner les « bons » candidats à la transplantation hépatique. Il convient

également de maintenir une survie à 5 ans supérieur à 70%, ce qui est le taux atteint dans le cadre de la transplantation hépatique hors CHC. Dans ce contexte, de nombreux travaux ont été effectués pour affiner le pronostic des patients greffés pour CHC, et définir des facteurs prédictifs de récidive post-transplantation. Le taux de récidive tumorale varie de 8 à 20% (8). La récidive post-transplantation hépatique est responsable de plus de 50% de la mortalité post-transplantation ; elle a un pronostic péjoratif avec une survie médiane de 6 mois post-récidive. Les facteurs prédictifs de récidive post-transplantation hépatique sont d'abord les caractéristiques morphologiques de la tumeur telles que le nombre de nodule, la taille de la plus grosse tumeur mais également le taux d'alpha-fœtoprotéine. L'alpha-fœtoprotéine est à ce jour, le seul marqueur biologique utilisé en routine pour aider à déterminer l'agressivité tumorale. Il reflète la différenciation tumorale ainsi que le risque de micro invasion vasculaire, tous deux marqueurs reconnus comme prédictifs de récidive post-transplantation hépatique (9). La Des-gamma-carboxy-prothrombine (DCP) dosée dans le sérum est aussi un marqueur intéressant qui présente une spécificité de 85% pour la présence d'une invasion micro vasculaire (10). Cependant, ce biomarqueur n'est pas utilisé en France en routine aujourd'hui. La réponse aux traitements d'attente comme la chimio-embolisation est également un facteur pronostique à considérer puisqu'il est associé à une meilleure survie à 5 ans chez les patients greffés (11).

A l'ère du développement de la génomique en oncologie, il convient de noter que l'avènement de marqueurs génétiques suscite beaucoup d'intérêt. La connaissance des caractéristiques moléculaires du CHC s'est affinée au cours des dernières années notamment grâce à l'analyse en masse de matériel tumoral et au séquençage de nouvelle génération (NGS). En effet, les critères diagnostiques du CHC se sont longtemps basées sur des méthodes non invasives évaluant les caractéristiques radiologiques de la tumeur. Ces caractéristiques sont l'existence d'un nodule hypervascularisé au temps artériel précoce (wash-in) avec lavage (wash-out) à la phase portale sur un scanner injecté avec triple acquisition. Désormais, de plus en plus, l'attitude de référence adoptée dans le diagnostic de CHC est la preuve histologique. Grace à la compréhension des mécanismes génétiques impliqués dans la genèse du CHC, un certain nombre d'outils à visée clinique ont vu le jour. Dans notre travail nous nous sommes intéressés à la place de l'ADN tumoral circulant (ADNtc) dans le champ du carcinome hépatocellulaire, et plus précisément comme marqueur prédictif de récidive post-transplantation hépatique. L'ADNtc est un biomarqueur sanguin innovant en oncologie. Son interprétation se base sur la connaissance des altérations génétiques d'une tumeur et plus précisément des mutations permettant de refléter la charge tumorale. Il convient d'exposer les principales altérations génétiques du CHC.

Le carcinome hépatocellulaire est une tumeur hétérogène présentant une genèse en multi-étapes où plusieurs altérations génétiques, notamment des mutations dites « driver », le plus souvent somatiques, entrent en jeu. Il existe un continuum dans la transformation maligne de lésions dysplasiques dans le contexte d'un foie fibreux. Ainsi les hépatocytes acquièrent des propriétés invasives, prolifératives et de survie.

Les mutations du promoteur de *TERT* (*TERTp*) sont les altérations moléculaires les plus fréquemment retrouvées dans le CHC (environ 60% des cas) (12). Les télomères sont des séquences répétées d'ADN situées à la fin de tous les chromosomes humains. Le rôle principal des télomères est de protéger les extrémités des chromosomes pour minimiser la perte d'ADN pendant les cycles de réplication cellulaire. Ces derniers sont synthétisés par la télomérase. Dans le CHC, les mutations activatrices du promoteur de *TERT* entraînent une hyperactivation de la télomérase, une instabilité génomique et une prolifération cellulaire. Les principales mutations sont des substitutions dans les hotspots situés 124 et 146 bases avant le début de l'ATG (13). Ces altérations se retrouvent également dans les lésions pré néoplasiques, et leur fréquence augmente en fonction du stade de la tumeur : les lésions dysplasiques de bas grade ont un taux de mutation proche de 6% alors que les lésions de haut grade ont un taux de 19% (12). La mutation du promoteur de *TERT* est un événement précoce dans la genèse du CHC et pourrait être considérée comme un évènement déclencheur de l'hépato carcinogenèse.

La voie de signalisation de p53 se retrouve altérée dans le CHC, comme dans de nombreuses tumeurs. Les mutations inactivatrices du gène suppresseur de tumeur p53 sont présentes dans près de 30% des cas (14). Ces mutations sont à l'origine d'une perte du contrôle du point G1/S du cycle cellulaire, et de l'acquisition de propriétés prolifératives. Les mutations de *TP53* sont caractéristiques de tumeurs peu différenciées, hautement prolifératives et présentant un pronostic souvent plus péjoratif. Les mutations de *TP53* sont souvent liées à une infection virale B (VHB) ou à une exposition à l'aflatoxin B1 notamment en Asie du sud-est et en Afrique (15).

Les altérations de la voie Wnt/β-caténine, liées notamment aux mutations activatrices de *CTNNB1* sont présentes dans environ 30% des CHC (16) et particulièrement dans les cirrhoses d'origine alcooliques. Elles sont aussi reconnues comme associées à un risque de transformation maligne des adénomes hépatocellulaires (17). La signalisation de la voie Wnt/β-caténine est activée par la liaison de Wnt à son récepteur transmembranaire *frizzled*, ce qui provoque l'arrêt de la dégradation de β-caténine par le protéasome, son accumulation dans le cytoplasme puis dans le noyau. Ainsi, en formant un complexe avec le facteur de transcription *TCF*, la cellule va acquérir des propriétés prolifératives et invasives (18). Les mutations de *CTNNB1* sont le plus souvent des substitutions ou des délétions, localisées sur les exons 3, 4, 7 ou 8 et sont exclusives des altérations de *TP53* (19).

D'autres voies de signalisation sont également impliquées dans la carcinogenèse hépatique. Notons la voie de signalisation *PI3K/AKT/mTor*, qui est activée dans environ 30 à 50 % des tumeurs, régule la prolifération cellulaire, l'apoptose et le métabolisme cellulaire (20). Aussi, les mutations activatrices de *NFE2L2* retrouvées dans plus de 5% des CHC codant pour le facteur de transcription *NRF2*, sont impliquées dans la réponse au stress oxydatif (20).

La connaissance acquise sur l'hépatocarcinogenèse, notamment dans le domaine de la génomique, a permis de développer des outils tels que l'utilisation de l'ADN tumoral circulant. L'analyse de ce dernier se base sur la détection et l'étude de cibles mutationnelles d'une tumeur. Il est de ce fait un biomarqueur précis et innovant.

L'ADNtc a été décrit pour la première fois dans les années 1970 par Leon et al. (21). Ils ont prouvé qu'il était quantifiable, et que son taux était plus élevé chez les patients atteints de cancer. L'ADNtc prend la forme de courts fragments d'ADN doubles brins d'une longueur inférieure à 200 paires de bases. Il provient de la libération spontanée d'ADN par la tumeur et surtout de la nécrose ou l'apoptose de cellules tumorales (22). Ainsi il peut être collecté par un simple prélèvement sanguin et récupéré au sein du plasma après centrifugation. 1 millilitre de plasma suffit à son analyse. L'ADNtc recueille toutes les altérations moléculaires caractéristiques de la tumeur ou des métastases : les mutations somatiques, mais également les anomalies épigénétiques telles que la perte d'hétérozygotie, les instabilités microsatellitaires ou la méthylation (23). Il a l'avantage d'être recueillis de façon peu invasive et de représenter l'ensemble de l'ADN tumoral. Il prend ainsi en considération l'hétérogénéité tumorale contrairement à la biopsie tumorale. L'analyse de l'ADNtc nécessite des techniques très sensibles telles que la digital PCR (dPCR) ou le séquençage à haut débit pour détecter un ratio allélique mutant/sauvage très faible. En effet, ce ratio représenté par la fréquence allélique d'un variant (VAF) est généralement proche de 1% (24).

Ainsi l'ADN tumoral circulant, en reflétant les caractéristiques moléculaires de la tumeur, pourrait servir de biopsie liquide. De même, par son analyse séquentielle et ses propriétés dynamiques, il a un rôle de biomarqueur permettant de suivre la maladie dans le temps.

Dans le domaine du CHC, plusieurs travaux se sont déjà penchés sur l'analyse de l'ADNtc mettant en lumière son apport dans le cadre du diagnostic et du pronostic. En effet, il est prouvé que le taux d'ADNtc est corrélé à la taille de la tumeur, au taux d'alpha-fœtoprotéine, au grade Edmonson ou encore à l'existence d'une extension microvasculaire ou métastatique (25) (26). De même, il est prouvé que ce taux évolue en fonction des thérapeutiques et reflète la charge tumorale. Ainsi il est possible de l'utiliser pour détecter une maladie résiduelle microscopique, une rechute précoce ou mettre en évidence une réponse au traitement (27). Il pourrait donc être un biomarqueur de meilleure qualité que l'alpha-fœtoprotéine. Dans notre étude, nous avons souhaité analyser et mesurer le taux l'ADNtc pour évaluer le risque de récidive tumorale post-transplantation hépatique pour carcinome hépatocellulaire, et le risque de sortie de liste d'attente pour progression tumorale.

Le projet « EFAPRE » est un PHRC national évaluant les facteurs prédictifs de récidive tumorale après transplantation hépatique pour CHC au sein d'une cohorte multicentrique prospective. Entre avril 2009 et décembre 2012, 371 patients ont été inclus et 279 ont été transplantés dans 17 centres de transplantation hépatique français. 38 patients ont présenté une récidive tumorale et 42 sont sortis de liste en raison d'une progression tumorale. Les patients ont tous bénéficié d'un prélèvement sanguin au moment de l'inscription sur liste de transplantation. Les cas ont été appariés à des témoins greffés et n'ayant pas récidivé durant un suivi médian de 5 ans. Les plasmas ont été recueillis et analysés au laboratoire INSERM « unité de génomique des tumeurs solides » (*U1138*). L'objectif était d'évaluer le risque de récidive tumorale dans

une population de patients greffés pour CHC, en analysant et quantifiant l'ADN tumoral circulant avant transplantation hépatique.

3. RESUME EN FRANCAIS

Introduction : Le carcinome hépatocellulaire (CHC), est la première tumeur maligne primitive du foie. Il représente un véritable problème de santé publique au niveau mondial puisqu'il représente la seconde cause de mortalité lié à un cancer dans le monde. Le traitement théorique idéal consiste en la transplantation hépatique. Dans cette étude, nous avons évalué l'apport de l'ADN tumoral circulant (ADNtc) pour affiner le pronostic des patients transplantés pour carcinome hépatocellulaire.

Méthodes : Nous avons collecté les échantillons plasmatiques d'une cohorte de 194 patients inscrits sur liste de transplantation hépatique pour CHC. Les plasmas ont été prélevés à l'inscription sur liste. Nous avons extrait l'ADN libre circulant du plasma, puis analysé l'ADN tumoral circulant par séquençage de « nouvelle génération ». Nous avons utilisé un panel ciblant les hotspots de mutations des gènes *TP53*, *TERT* promoteur, *CTNNB1*, *PIK3CA* et *NFE2L2*, qui sont les gènes les plus souvent mutés dans le CHC. La détectabilité de l'ADNtc et sa quantification ont été analysés pour prédire le risque de récidive post-transplantation ou de sortie de liste pour progression tumorale.

Résultats : 42 patients sortis de liste pour progression tumorale ont été appariés à 90 patients transplantés. 38 patients ont récidivé avec un délai de suivi de 5 ans, et ont été appariés à 81 patients transplantés et n'ayant pas présenté de récidive tumorale. Les patients ayant récidivé avaient dans l'ADN plasmatique un taux de mutation de *TP53*, *TERTp* et *CTNNB1* plus élevé que les patients contrôles mais non significatif (p=0.49 ; p=0.93 ; p=0.09 respectivement). Aucune mutation de *PIK3CA* ou *NFE2L2* n'a été mise en évidence au sein de ce groupe. La fréquence des mutations de *TP53*, *TERTp*, et *CTNNB1* n'était pas différente entre les patients ayant récidivé et les patients contrôles (p=0.13 ; p=0.74 ; p=0.61 respectivement). Chez les patients sortis de liste pour progression tumorale, il n'y avait pas de différence significative en terme de taux de mutation des gènes *TP53*, *TERTp* (p=0.92 ; p=0.92 respectivement) ni de la fréquence des mutations de *TP53* et TE*RTp* (p=0.13 ; p=0.72 respectivement). Aucune mutation de *CTNNB1*, *PIK3CA* ou *NFE2L2* n'a été mise en évidence chez les patients sortis de liste. Dans la cohorte globale de patients nous avons mis en évidence une association significative entre la fréquence des mutations de *TERTp* et le nombre de nodules (p=0.035), et entre la fréquence des mutations de *CTNNB1* et l'âge (p=0.014). Enfin il existait une tendance entre la fréquence des mutations de *TERTp* et le taux d'alpha-fœtoproteine (p=0.098) et l'existence d'une NASH (p=0.061). De même, le taux de mutation de *CTNNB1* était associé au diamètre de la plus large tumeur (p=0.168) et au taux d'alpha-fœtoproteine (p=0.127) de façon non significative.

Conclusion : L'ADNtc est un marqueur biologique intéressant mais qui ne semble pas aider à affiner le pronostic des patients inscrits sur liste de transplantation hépatique pour CHC, que ce soit pour la prédiction de progression tumorale sur liste, ou celle de récidive tumorale après transplantation. L'absence de valeur pronostique est très certainement due à l'hyper-sélection de cette population.

4. ARTICLE

Circulating tumor DNA as prognostic marker of tumor recurrence/progression during the follow-up of patients on the waiting list of liver transplantation for hepatocellular carcinoma: a multicenter prospective study

O. Msika, B. Noblet, N. Oubaya, S. Imbeaud, JC. Nault, J. Zucman-Rossi, T. Decaens.

4.1 Abstract

Background: Hepatocellular carcinoma, is the first primary malignant tumor of the liver. It represents a real public health problem at the global level as it is the second leading cause of cancer-related mortality worldwide. The ideal theoretical treatment is liver transplantation. In this study, we assessed the prognostic power of circulating tumor DNA (ctDNA) in a prospective cohort of patients listed for liver transplantation for hepatocellular carcinoma.

Methods: Prospective cohort of 371 patients listed for liver transplantation for hepatocellular carcinoma. Plasma samples were collected prospectively for all patients at the time of listing. We analyzed plasma samples from a cohort of 194 patients chosen on a case-control study basis. We extracted cell free DNA from the plasma and then analyzed ctDNA by "next generation" sequencing. We used a panel targeting hotspot mutations in *TP53*, *TERT* promoter, *CTNNB1*, *PIK3CA* and *NFE2L2* genes, which are the most frequently mutated genes in HCC. The detectability of ctDNA and its quantification were analyzed to predict the risk of post-transplant recurrence or drop-out for tumor progression.

Results: 42 patients dropped out from the waiting list for tumor progression were matched to 90 transplant patients. In parallel, 38 patients experienced tumor recurrence within a 5-year follow-up period, and were matched to 81 transplanted patients who did not have recurrence. Patients who recurred had a higher but not significant mutation rate in plasma DNA for *TP53*, *TERTp* and *CTNNB1* than controls (p=0.49; p=0.93; p=0.09 respectively). No *PIK3CA* or *NFE2L2* mutations were found in this group. The variant allele frequency (VAF) of *TP53*, *TERTp*, and *CTNNB1* mutations was not different between recurrence and control patients (p=0.13; p=0.74; p=0.61 respectively). In patients dropped out from the list for tumor progression, there was no significant difference in the mutation rate of *TP53*, *TERTp* (p=0.92; p=0.92 respectively) or in the frequency of *TP53* and *TERT*p mutations (p=0.13; p=0.72 respectively). No mutation in *CTNNB1*, *PIK3CA* or *NFE2L2* were found in the drop-out patients. Finally, in the overall cohort of patients we found a significant association between the *TERTp* mutations VAF and the number of nodules (p=0.035), and between the *CTNNB1* mutations VAF and age (p=0.014). Finally, there was a trend between the *TERTp* mutations VAF and alpha-fœtoprotein level (p=0.098) and the existence of NASH (p=0.061). Similarly, the *CTNNB1* mutation VAF was associated with the diameter of the largest tumor (p=0.168) and alpha-fœtoprotein level (p=0.127) but is not significant.

Conclusion: ctDNA is an interesting biological marker but failed to help the refinement of tumor progression during the waiting time or tumor recurrence after liver transplantation in a large cohort of patient listed for LT for HCC. The absence of prognostic factor is probably due to strict selection of these patients.

Key words: *Hepatocellular carcinoma, circulating tumor DNA, liver transplantation, Next generation sequencing, biomarker, cell free DNA, prognosis*.

4.2 Abbreviation List

- BCLC: Barcelona Clinic Liver Cancer
- COSMIC: Catalogue of Somatic Mutations in Cancer
- CT: Computed Tomography
- CTNNB1: Catenin beta1
- ctDNA: circulating tumor DNA
- cfDNA: cell free DNA
- DFL: Drop-out from list
- dPCR: digital polymerase chain reaction
- HBV: Hepatitis B Virus
- HCC: Hepatocellular Carcinoma
- HCV: Hepatitis C Virus
- LT: Liver transplantation
- MRI: Magnetic Resonance Imaging
- NASH: Non-Alcoholic Steato-Hepatitis
- NGS: Next Generation Sequencing
- PCR: polymerase chain reaction
- PBMC: Peripheral Blood Mononuclear Cells
- RFA: Radiofrequency Ablation
- TERT: Telomerase Reverse Transcriptase
- TP53: Tumor protein 53
- VAF: Variant Allele Frequency

4.3 Introduction

Hepatocellular carcinoma (HCC) is the first malignant tumor of the liver. It is a major public health problem in the world as HCC is the sixth most common tumor and the fourth most common cause of death from cancer (28). In 2030, HCC incidence is expected to increase by 35% compared to 2005 (29). It occurs most often in the context of chronic liver disease or cirrhosis. This tumor is one of the few that can be treated by transplantation, which is the ideal theoretical treatment. Selection criteria for liver transplantation (LT) are today based on morphological criteria such as tumor size, number of nodules, but also on alpha-fetoprotein level (5). These criteria allow a 5-year survival rate of more than 70%. However, the benefit of this therapy is conditioned by tumor recurrence after liver transplantation. Today this risk is estimated at approximately 8-20% (8). Given the shortage of organs, more precise criteria should be defined for the selection of patients allowing a real survival benefit. Several parameters are now recognized as markers of survival in post-liver transplantation, including histologic grade, tumor differentiation (30), vascular invasion. Other parameters such as response to neoadjuvant treatments (31), waiting time before LT (32) or immunosuppression used in post-transplantation (33) have shown an interest in the prediction of tumor recurrence. Currently, it is appropriate to define more precise and more efficient predictive markers for post-liver transplantation recurrence.

Thanks to the technical advances realized in the field of DNA sequencing, in particularly in NGS (Nest Generation Sequencing), the mutational landscape of HCC is now well described. Recent studies have shown by whole exome tumor sequencing that the most common driver mutations were located in the telomerase reverse transcriptase (*TERT*), tumor protein p53 (*TP53*), and beta-catenin (*CTNNB1*) genes (34). Understanding tumor biology and genomics have made possible to develop genomics biomarkers, as circulating tumor DNA (ctDNA). In the last years, ctDNA has gained importance as a tool for non-invasively characterizing molecular alterations of a tumor. ctDNA comes from spontaneous release by the tumor or from apoptosis and necrosis of tumor cells (35). It is measured by the fraction of the cell free DNA harboring a specific mutation in a background of wild type cfDNA. It is more easily available than a biopsy and it collects all the genetic abnormalities. ctDNA would provide a genetic profile of the tumors, circumventing tumor heterogeneity (36). Through quantification, it reflects the tumor burden and provides dynamic information on tumor evolution. There is evidence that ctDNA levels are higher in cancer patients (21) and can help to monitor response to treatment (37). Thus, it is an effective prognostic marker. In fact, in the context of HCC, ctDNA levels are correlated with microvascular invasion and the risk of metastatic extension (26). But no study has shown the interest of circulating tumor DNA sequencing in refining the prognosis of HCC transplant patients.

Thus, in this study, we wish to contribute, using ctDNA analyze, to precise the risk of tumor recurrence after liver transplantation for HCC. The main objective was to

evaluate the contribution of ctDNA as a predictive marker of tumor recurrence or drop-out from waiting list for tumor progression.

4.4 Materials & Methods

Study design and patient's selection

Between April 2009 and December 2012, 371 patients were prospectively enrolled in the "EFAPRE" research cohort. The inclusion criteria were being HCC diagnosed histologically or according to Barcelona criteria (38), being of age, having consent and being eligible for liver transplantation. 279 patients were transplanted. They were recruited from 17 French university hospitals participating to the study (Paris, Lille, Strasbourg, Rennes, Besançon, Bordeaux, Lyon, Grenoble, Toulouse, Marseille, Nice). Each patient had a 10 mL blood sample, radiological imaging by CT and/or MRI at the registration on the waiting list. The serum bank was fed every 3 months until the liver transplant. Only the blood samples taken at the time of listing (M0) was analyzed. After LT, patients were followed every 6 months for 5 years. They had an alpha-fetoprotein dosage and a CT scan every 6 months during the two first year after transplantation. All patients who experience tumor recurrence (n=38) or who were dropped out of the waiting list for tumor progression (n= 42) were included. Recurrence was defined according to imaging data. Clinical, biological, imaging and pathological data were prospectively collected in a CRF. Informed consent of patients was obtained for all patients according with French legislation. The study was approved by the Ethics Committee.

Patients dropped out from list for tumor progression (n= 42) were matched to controls (n= 81) (transplant patients with or without recurrence) in a 1:2 up to 1:4 ratio. During follow-up, 38 patients developed tumor recurrence. These patients were also matched to transplant patients who did not present tumor recurrence (n=81) with a median follow-up time of 5 years (*Figure 1*). Matching criteria were: age (±5 years, widened to ±10 years and ±15 years to avoid losing cases), sex, tumor size (±10 mm widened to ±20) and number (±1 widened to ±2 and ±3), alpha-fœtoprotein level (<100 vs. ≥100), length of waiting time (<6 months/[6-18] months/>18 months), and type of bridging treatments before listing or while waiting (chemoembolization, radiofrequency ablation or surgery) and number of treatment during the waiting time. For recurrence group, follow-up time after transplant of controls had to be greater than the follow-up time of their respective cases.

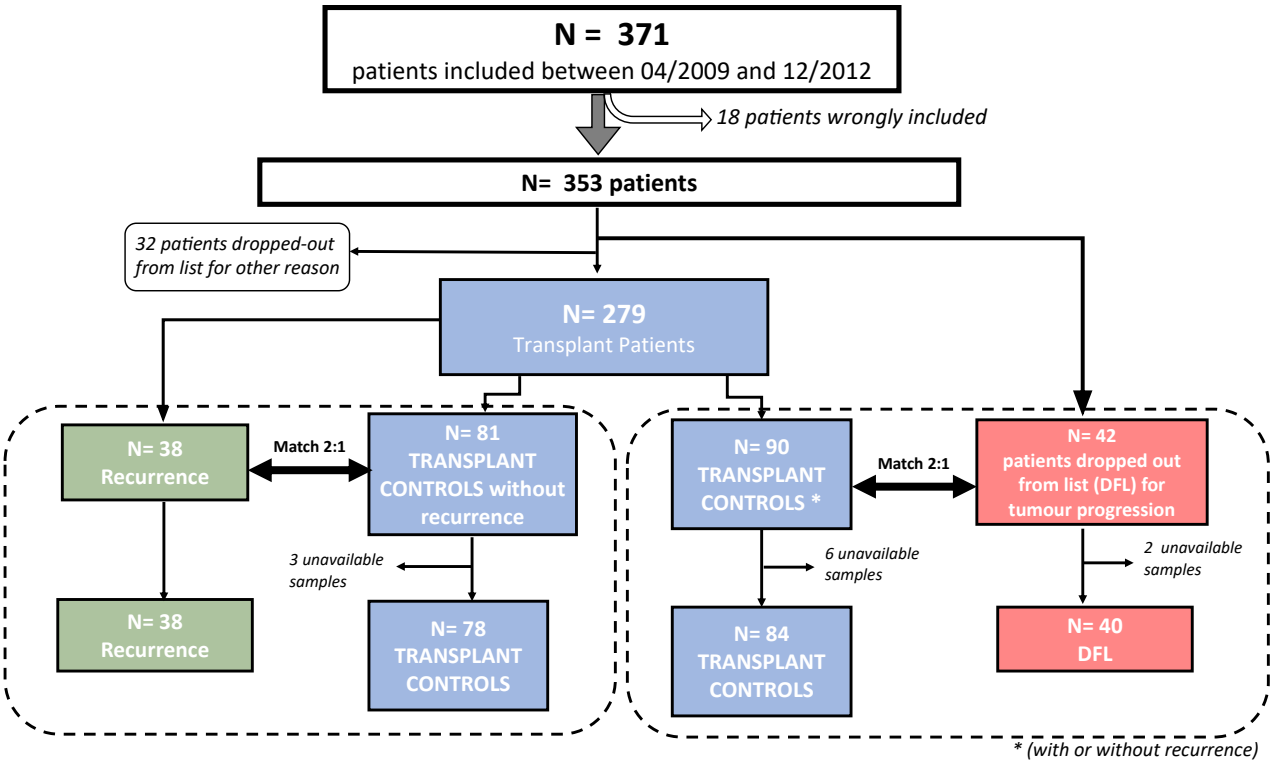

Figure 1: Study Flowchart

cfDNA extraction and quantification

1 mL of frozen plasma was used to extract cfDNA using DNA extractor kit for plasma (Maxwell® RSC ccfDNA Plasma Kit; Ref. AS1480 of Promega, Charbonnières les Bains, France) according to manufacturer's instructions. This is a paramagnetic ball purification technology that efficiently bind cfDNA to the paramagnetic particle in the well of a prefilled cartridge. 60µL of Elution Buffer are added in each elution tube. This will give a final elution volume after processing of approximately 50µL. The extraction process was performed with the Maxwell® RSC instrument to purify circulating cell-free DNA (ccfDNA). Extracted DNA eluent was stored at -20 ° C.

DNA was quantified using the Quantifluor® dsDNA System (ref E2670; Promega, Charbonnières les Bains, France) according to the manufacturer's manuals. This method is based on the measurement of fluorescence. QuantusTM Fluorometer was used to measure fluorescence, with the dsDNA protocol after calibration with standards. Fluorometer is capable of measuring excitation and emission at the appropriate wavelengths. There was no DNA quality analysis due to the low amount of nucleic acid.

Selection of a panel of HCC specific mutations

Plasmas samples were subjected to sequencing for 4 protein-coding genes involved in HCC (*CTNNB1*, *TP53*, *PIK3CA* and *NFE2L2*) and the promoter region of

TERT. We designed a target panel of hotspots. This panel consisted of 61 amplicons, with 61 primers pairs targeting exons 3,7 and 8 of *CTNNB1*, hotspots 124 and 146 of *TERT* promoter, 9 exons of *TP53* (1 to 10 except exon 2), exon 2 of *NFE2L2* and exon 10 and 23 of *PIK3CA*. The choice of these primers is based on data provided by the COSMIC (Catalogue of Somatic Mutations in Cancer) database and the local database. The tool BioMart of ensembl and NCBI was used to extract biological transcripts. Primers (forward and reverse) were defined for each amplicon with use of the Primer3web V4.1.0 tools(39). Chosen primers had a "GC" ratio less than 70% and similar ones for those in forward and reverse, furthermore, primers Tm need to be close to 58 to 63° C and similar for all. Generation of the primers was done thanks to the PrimerDesign.R software.

Library Sequencing and target enrichment

Circulating free DNA was sequenced using MiSeq® sequencing platform (*Illumina Inc*). Despite the low concentrations of DNA extracted in the plasma, sequencing is feasible. An equal volume of 12µL of each sample of extracted cfDNA was used for libraries preparation regardless of their concentration. Given the limited number of amplicons, the multiplexing was done manually on a 384-well plate, by the constitution of 3 mixes of 15 primers and one of 16 primers.

Enrichment was performed by PCR on multiplex thermal cycler. Genomic DNA was amplified with adding of dimethylsulphoxide (DMSO) 2,5%/glycerol 5%. A touchdown PCR was done in, GeneAmp® PCR 9700 (Applied Biosystems, Courtaboeuf, France), with the following parameters: an initial denaturation at 94°C during 15 minutes to activate the DNA polymerase, and 50 cycles of 94°C for 30 sec, 62°C for 30 sec, 72°C for 1 minutes (decreasing annealing temperature by 1°C per cycle) and ending with 72°C for 5 minutes.

Products of PCR1 were purified using Agencourt AMPure® XP magnetic beads (Beckman-Coulter, Villepinte, France). The second PCR reaction indexing and adding P5/P7 primers was done on a GeneAmp® PCR 9700 system (Applied Biosystems, Thermo Fisher Scientific) with the following program: 15 minutes at 94°C, 12 cycles: 15 sec at 94°C, 30 sec at 60°C and 2 minutes at 60°C and then 5 minutes at 72°C.

Each PCR 2 products were purified with Agencourt AMPure® XP magnetic beads with use of 20µL of AMPure XP beads and PCR products are quantified on an Applied Biosystems® (ThermoFisher) 7900HT Fast Real-Time PCR System with Syber Green® and probes targeting P5/P7 sequences. The PCR reaction was done with the following program: 10 minutes at 95°C, 30 cycles: 15 sec at 95°C and 1 minute at 60°C. The results are analyzed with the SDS 2.4 software.

Libraries were quantified using KAPA® Library Quantification Kits (Roche, Rosny-sous-Bois, France). PCR reaction is done with the following program: 5 minutes at 95°C, 35 cycles: 30 sec at 95°C and 45 sec at 60°C.

The library was denatured with freshly diluted NaOH and the reaction is stopped by adding Hybridization Buffer (HT1). 600µl of the denatured pool diluted at

6pM are deposited in the thawed cartridge ready to be loaded into the MiSeq (*Illumina Inc*). MiSeq System is fitted with a Real-Time Analysis software which runs on the instrument control computer. During a sequencing run, it extracts intensities from images to perform base calling, and assigns a quality score to the base call. Sequencing was performed with paired-end reads using the Illumina Paired End v2 kit with an average read depth of >15,000X per amplicon except for exons of *NFE2L2* and *PIK3CA* for whom the average coverage was less than 1000X. The representation of each sample in the library pool is generated via the Illumina Sequence Analysis Reporter Software in the form of FATSQ file. The "BAM" (Binary Alignment Map) files of each library are merged for each sample. The coverage is calculated using the GATK Depth of Coverage tool (Genome Analysis Toolkit, Broad Institute) from the merged BAM files. Variant are classified as "low quality", "rare polymorphism", "frequent polymorphism", "somatic or germline" depending on the quality of the sequence, the number of read altered and the frequency of the variants.

Bioinformatical analysis and Variant Calling

The variant allele fraction (VAF) was calculated as the proportion of cfDNA harboring the variant in a background of wild-type cfDNA. All fastq data were trimmed for adapters and primers sequence (25 bp) using cutadapt 2.10 and reads were mapped to human genome 19 (GRCh37 hg19) using bwa-mem (v 0.7.5a-r405). Alignment and BAM metrics were controlled using Picard Tools (v 2.23.1). Reads were assigned to the corresponding amplicon, followed by a per position (A, C, G, T) coverage for each amplicon using a NG-TAS javascript(40). Mutation calling was done in each amplicon and defined as a sequence differing from the reference genome. For each position, a score was attributed as the proportion of the amplicons set displaying the variation. A mutation called in 2 out of a 3 amplicons set was considered in subsequent analysis. We then estimated the error rate for each variant call using a series of 42 control liver samples (with no known liver tumor). Reference strand biased and noisy variant positions were computed using the R Plasma Mutation Detector (v 1.7.2)(41). The significant set of mutations corresponded as the upper bound of a robust boxplot of the finite logit p-value using a binary logistic regression. Mutations were annotated with Ensembl Variant Effect Predictor (VEP, v98(42)) and OncoKB (1.1.2,(43)) so we kept only coding variants (including promoter regions) and excluded those variants already known with frequency > 0.01% in gnomAD (Genome Aggregation Database, (44)) population.

Statistical Analysis

Categorical variables were quoted as the number (percentage) and quantitative variables were described as the mean ± standard deviation or the median [interquartile range (IQR)], as appropriate. We looked for factors associated with recurrence or drop out from the list, respectively, using mixed-effects logistic regression with a random intercept, accounting for matching. The results were

expressed as an odds ratio (OR) [95% confidence interval (CI). VAF values were categorized into terciles or quartiles (depending on the amount of non-zero data available) with the zero-value considered as the reference level. An unmatched analysis was performed to assess factors associated with mutations (at least on mutation or presence of a specific mutations on *TERT* gene, *CTNNB1* or *TP53*) with the means of Chi square tests of Fisher exact tests, as appropriate, for qualitative variables and Student t tests or Mann-Whitney tests, as appropriate, for quantitative variables. Factors associated with cfDNA concentration and VAF of the genes with mutations were searched in an unmatched analysis using Spearman correlation coefficient tests for quantitative variables, Mann-Whitney tests for binary variables and Kruskal-Wallis tests for non-binary variables. All significance tests were two-tailed, and the threshold for statistical significance level was set to 5%. Statistical analyses were conducted using Stata 16.0 (StataCorp, College Station, TX, USA)) and GraphPad Prism Software.

4.5 Results

4.5.1 Recurrence vs control patients

4.5.1.1 Patients' demographics

Clinical characteristics of patients are presented in Table 1. The mean age of patients at listing inscription was comparable between the two groups; 58.7 years in controls and 57.8 years in patients with recurrence. The majority of patients were male (95.1% in the control group and 100% in the recurrence group). The main etiology of liver disease was alcohol (65.4% in control patients and 65.8% in patients with recurrence), followed by HCV infection and NASH. The mean number of nodules was 1.9 and 2.3 in the controls and recurrence group respectively. The mean largest tumor size was 22.1 mm in the control group and 24.4 mm in the recurrence group. The majority of patients in both groups had Child A cirrhosis. In both groups, the majority of patients had received HCC treatment prior to listing, most often chemo-embolization (55.6% in control group and 63.2% in recurrence group) *(Table 1).*

	Control N=81	**Recurrence** N=38
Age at listing inscription mean (±SD)	58.7 (±5.5)	57.8 (±5.3)
Male gender n(%)	77 (95.1%)	38 (100.0%)
Etiology		
HBV infection n(%)	6 (7.4%)	1 (2.6%)
HCV infection n(%)	23 (28.4%)	15 (39.5%)
Alcohol n(%)	53 (65.4%)	25 (65.8%)
NASH n(%)	11 (13.6%)	10 (26.3%)
Hemochromatosis n(%)	5 (6.2%)	2 (5.3%)
Metabolic syndrom n(%)	7 (8.6%)	7 (18.4%)
Conjuguated bilirubin level (µM) mean (±SD)	16.2 (±26.4)	13.3 (±21.3)
Creatinine (µM) mean (±SD)	75.3 (±20.1)	68.1 (±12.5)
Albumin (g/L) mean (±SD)	34.1 (±5.9)	37.0 (±5.4)
PT (%) mean (±SD)	66.6 (±17.0)	77.4 (±18.3)
Child Pugh class n(%)		
A	44 (55.0%)	28 (75.7%)
B	24 (30.0%)	7 (18.9%)
C	12 (15.0%)	2 (5.4%)
MELD Score mean (±SD)	12.2 (±4.8)	9.2 (±3.0)
Number of nodules mean (±SD)	1.9 (±1.2)	2.3 (±1.3)
Diameter of the largest tumor (mm) mean (±SD)	22.1 (±12.1)	24.4 (±9.7)
Alpha-foetoprotein (ng/mL) mean (±SD)	94.7 (±558.8)	35.4 (±77.5)
Alpha-foetoprotein (ng/mL) n(%)		
<100	76 (93.8%)	35 (92.1%)
≥100	5 (6.2%)	3 (7.9%)
Treatment before transplantation listing		
Chemoembolization n(%)	45 (55.6%)	24 (63.2%)
Thermoablation n(%)	15 (18.5%)	4 (10.5%)
Hepatic resection n(%)	6 (7.4%)	6 (15.8%)

Table 1: Characteristics of recurrence patient populations and their matched controls

4.5.1.2 cfDNA concentration determination

The cell free DNA is the whole tumoral or non-tumoral plasma DNA. The mean level of circulating cell free DNA was 0.193 ng/µL in the recurrence group and 0.187 ng/µL in the control group. There was no statistically significant difference between the two groups (OR: 1.20; 95%CI:0.31;4.57 p=0.79) (*Figure 2*).

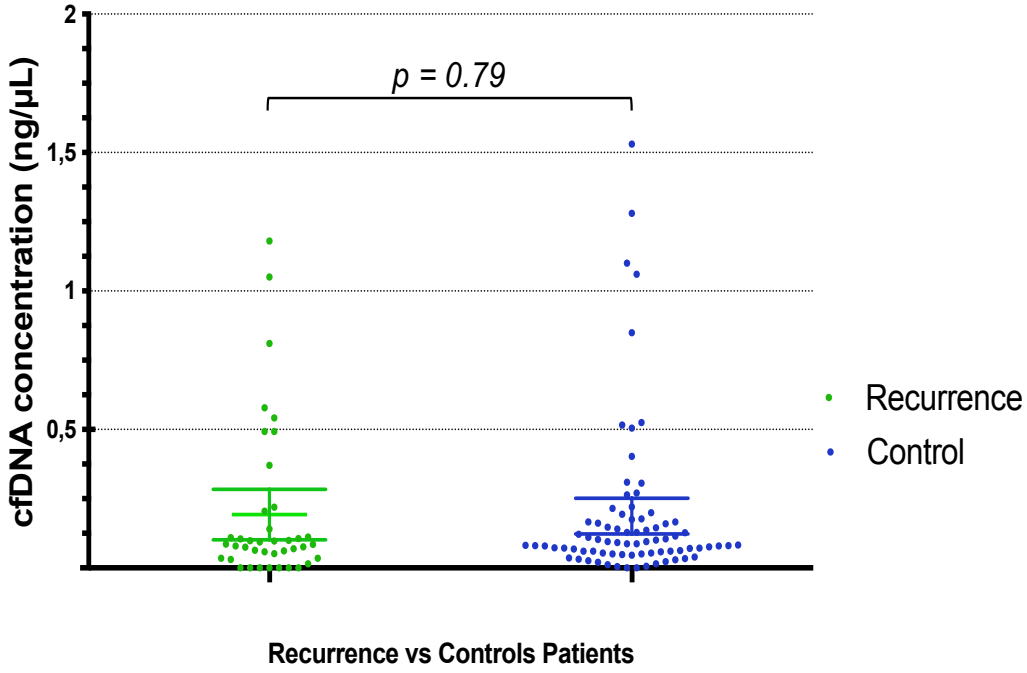

Figure 2: cfDNA concentration determination in recurrence and control patients.

4.5.1.3 Somatic Mutation detection

ctDNA was detected in 28.9% of patients with recurrence (11/38) and in 21.8% of control patients (17/78) (OR: 1.33; 95%CI: 0.54;3.28, p=0.54). The detectability of ctDNA was defined by the presence of at least one detectable somatic mutation. *TP53* mutation was detected in 15.8% patients with recurrence (6/38) and in 11,5% control patients (9/78) (OR: 1.48; CI95%: 0.49;4.53, p=0.49) *(Figure 3)*. *TERTp* was mutated in 13.2% patients with recurrence (5/38) and in 10,3% controls patients (8/78) (OR: 1.06; CI95%: 0.30;3.78, p=0.93). *CTNNB1* mutation was identified in 10.5% (4/38) vs 2.6% (2/78) in recurrence and control patients respectively (OR: 4.61; CI95%: 0.80;26.40, p= 0.09). All *TERTp* mutation were c.-124C>T, located in the 5'flank DNA region. Mutations of *CTNNB1* and *TP53* were all missense mutations. There was no mutation detected in PIK3CA and NFE2L2.

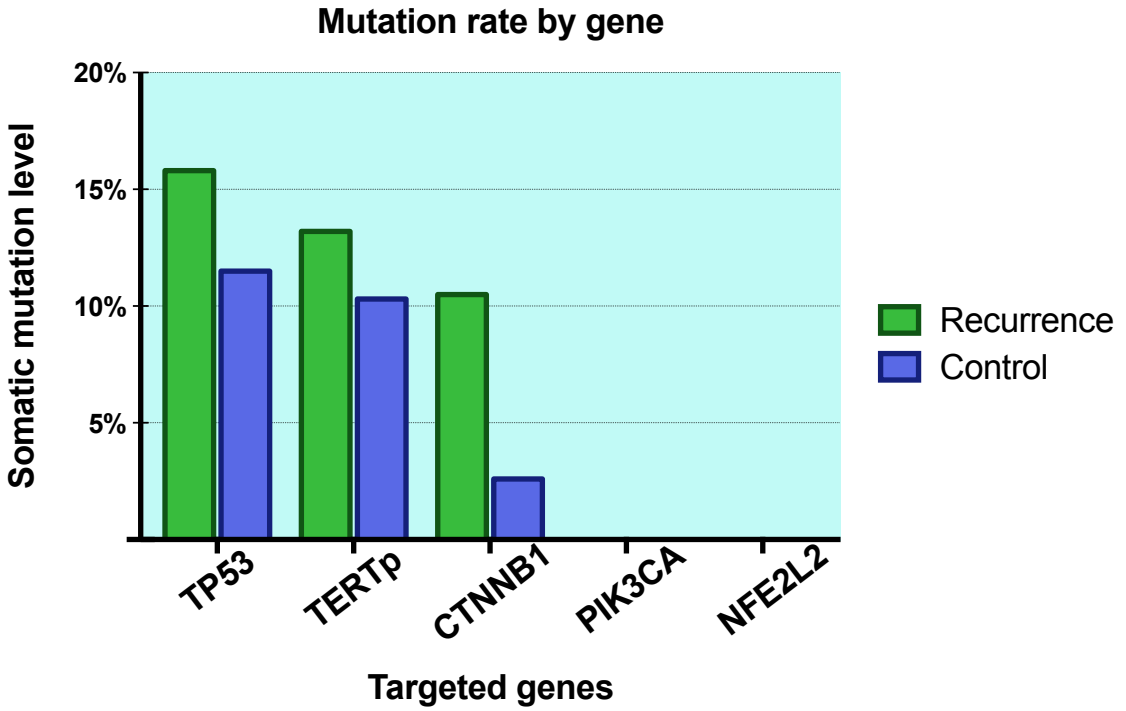

Figure 3: Rate of detected mutations in recurrence and control groups

Thus, there was no association between the detectability of ctDNA and the risk of recurrence. There was also no mutation associated with the risk of recurrence following liver transplantation, although there is a clear trend for the *CTNNB1* mutation. In the recurrence group, 73% of patients had no mutated gene, 16.2% had one mutated gene (OR: 0.90; 95%CI: 0.32;2.58, p=0.85) and 10.8% had two mutated genes (OR: 4.52; 95%CI: 0.78;26.18, p=0.09). Having two mutated genes was associated with poorer prognosis, but this association was not significant. No patient had mutations in all three genes *TP53*, *TERTp* and *CTNNB1*. In the recurrence group, 2 patients had concomitant *TP53* and *CTNNB1* mutations. This is unusual as it is usually considered that mutations in these two genes are mutually exclusive.

No association was found between the concentration of cfDNA extracted from plasma and the detectability of ctDNA. Patients with detectable ctDNA had a mean cfDNA concentration of 0.21 ng/µL versus 0.19 ng/µL in patients without ctDNA (p=0.68). The number of mutated genes per patient was also not associated with cfDNA concentration (p=0.78).

4.5.1.4 Quantification of circulating tumor DNA

ctDNA was quantified by measuring the level of the mutated allele within the total circulating DNA. This rate is represented by the VAF, and is determined for each gene respectively. For *TP53* gene, the mean VAF of patients who recurred was 2.2% (range: 1.05-4.13%), and 1.3% (range: 0.74-2.55%) in the control group (p=0.13). The mean VAF for *TERTp* mutations was 1.07% (0.2-2.94%) in the recurrence group versus 0.88% (0.28 - 2.67%) in the control group (p=0.74). For the *CTNNB1* gene, the mean VAF was lower in the recurrence group than in the control group, as it was 1.9% (0.62-4.92%) in the recurrence group versus 2.89% (1.39-4.39%) in the control group. This difference was not statistically significant (p=0.61).

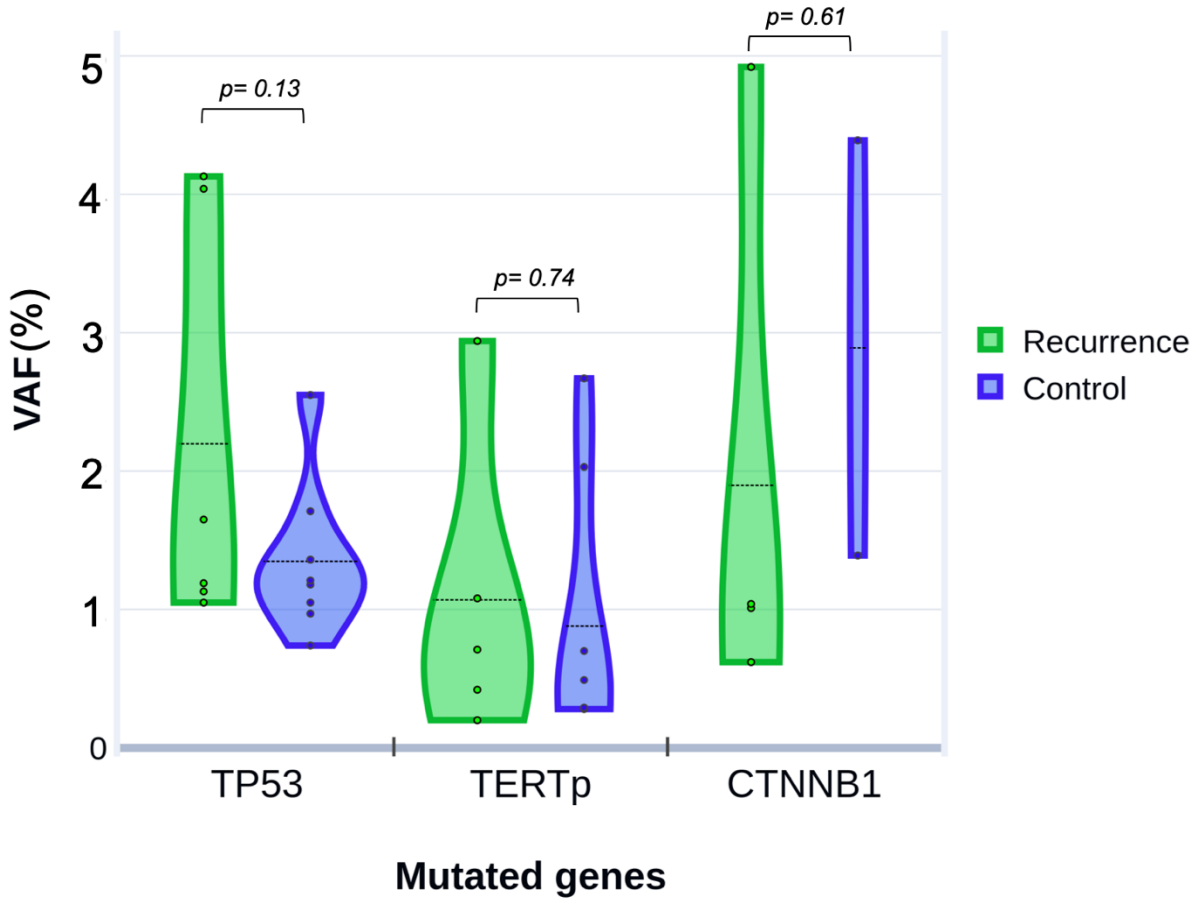

Figure 4: Distribution of VAF according to the mutations of the different target genes in the recurrence versus control groups.

4.5.2 Dropped out from list vs control patients

4.5.2.1 Patients' demographics

Clinical characteristics of patients are presented in Table 2. In both groups, the mean age at listing was comparable as 58.2 years in the control group and 57.2 years in the drop-out group. The majority of patients were male. 75% of patients in the control group had alcohol liver disease, 72.5% in the drop-out group. The average number of nodule was 1.70 and 2.08 in the control and drop-out groups respectively, in accordance with the Milan criteria. The mean diameter of the largest tumor was 24.9 mm (±12.7) in the control group versus 26.5 mm (±15.9) in patients dropped out from list for tumor progression. In both groups cirrhosis was most often ranked A (60.5% in the control group and 70.3% in the relapse group), and the mean MELD scores were comparable 11.1 and 11.0 respectively. The majority of patients experienced pre-listing chemoembolization, and those in both groups *(Table 2).*

	Control N=84	**Dropped-out from list** N=40
Age at listing inscription mean (±SD)	58.2 (±5.4)	57.2 (±6.4)
Male gender n(%)	76 (90.5%)	34 (85.0%)
Etiology		
HCV infection n(%)	22 (26.2%)	10 (25.0%)
HBV infection n(%)	12 (14.3%)	6 (15.0%)
Alcohol n(%)	63 (75.0%)	29 (72.5%)
NASH n(%)	12 (14.3%)	7 (17.5%)
Hemochromatosis n(%)	2 (2.4%)	0 (0.0%)
Metabolic syndrom n(%)	16 (19.1%)	6 (15.4%)
Conjuguated bilirubin level (µM) mean (±SD)	10.4 (±12.0)	12.3 (±12.6)
Creatinine (µM) mean (±SD)	82.0 (±67.4)	74.9 (±20.3)
Albumin (g/L) mean (±SD)	35.2 (±5.8)	34.0 (±5.4)
PT (%) mean (±SD)	72.3 (±17.9)	70.0 (±16.0)
Child Pugh class n(%)		
A	49 (60.5%)	26 (70.3%)
B	28 (34.6%)	9 (24.3%)
C	4 (4.9%)	2 (5.4%)
MELD Score mean (±SD)	11.1 (±4.2)	11.0 (±3.4)
Number of nodules mean (±SD)	1.70 (±1.0)	2.08 (±1.1)
Diameter of the largest tumor (mm) mean (±SD)	24.9 (±12.7)	26.5 (±15.9)
Alpha-fœtoprotein (ng/mL) mean (±SD)	43.0 (±110.6)	69.7 (±154.6)
Alpha-fœtoprotein (ng/ml) n(%)		
<100	77 (91.7%)	34 (85.0%)
≥100	7 (8.3%)	6 (15.0%)
Treatment before transplantation listing		
Chemoembolization n(%)	51 (56.7%)	21 (50.0%)
Thermoablation n(%)	20 (22.2%)	14 (33.3%)
Hepatic resection n(%)	9 (10.0%)	2 (4.8%)

Table 2: Clinical characteristics of patient's dropped out from the waiting list for tumor progression and matched controls

4.5.2.2 cfDNA concentration determination

The mean level of circulating cell free DNA was 0.310 ng/µL in drop-out group and 0.563 ng/µL in the control group. There was no statistically significant difference between the two groups (OR: 0.94; 95%CI: 0.73;1.21, p=0.63) (*Figure 5*).

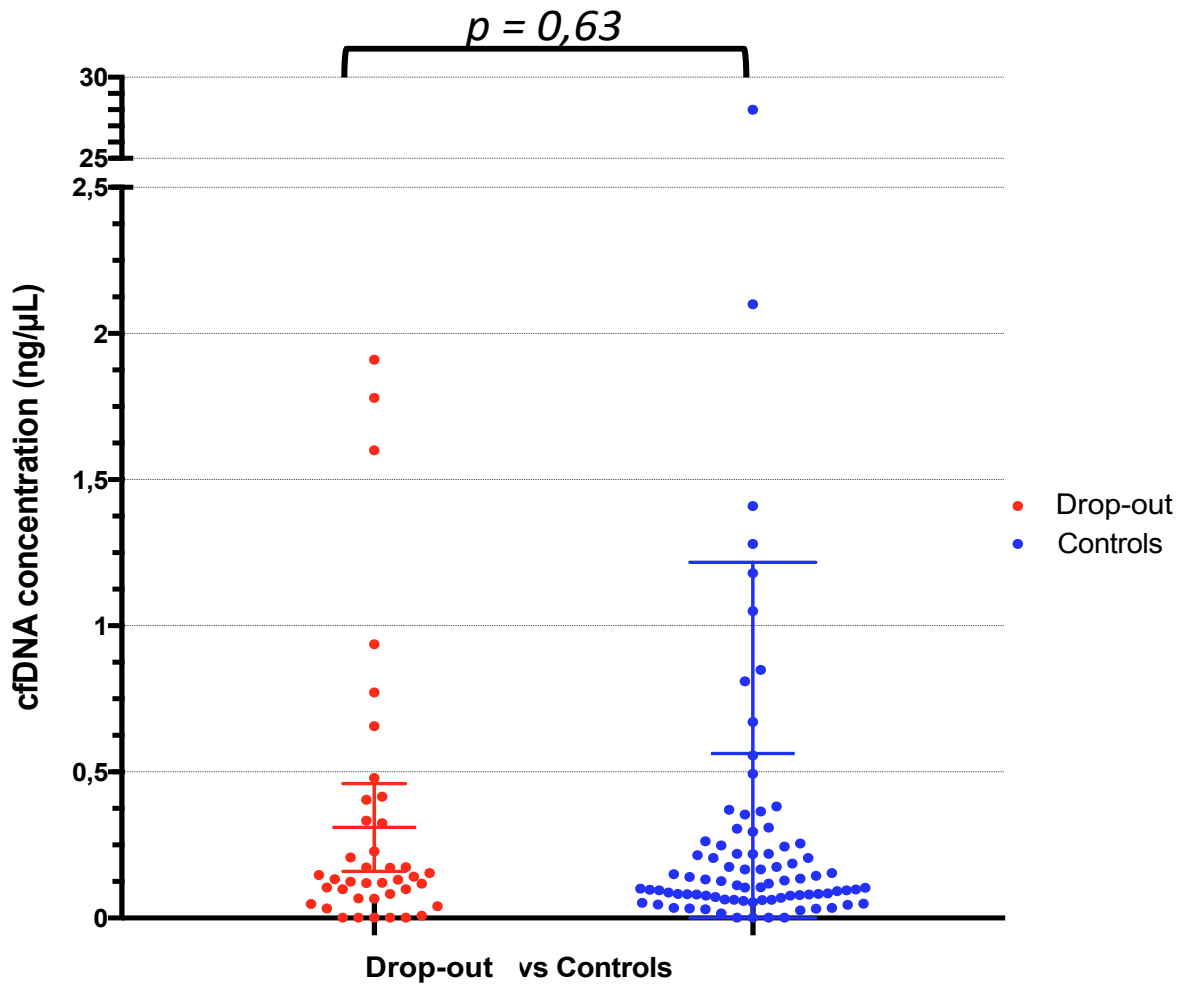

Figure 5: cfDNA concentration determination in drop-out and control patients.

4.5.2.3 Somatic Mutation detection

ctDNA was detectable in 25% of patients dropped out from list for tumor progression (10/40) and in 26.2% of control patients (22/84). *TP53* was the most frequently mutated gene in the drop-out group, as 15% of patients (6/40) had a *TP53* mutation. In the control group, *TP53* was detected as mutated in 14.3% of patients (12/84) (OR: 1.06; 95%CI: 0.37;3.06, p=0,92) (*Figure 6*). All mutation were also missense mutations. One patient had a mutation p.Arg249Ser which is known as associated with aflatoxin B1 intoxication. One patient had 3 mutations in *TP53* gene which was associated with a high VAF. *TERTp* mutation was identified in 12.5% of drop-out patients (5/40) and in 11.9% of control patients (10/84). This difference was

also not statistically significant (OR: 1.06 95%CI: 0.34;3.33, p= 0,92). Contrary to the group of patients who have recurred, we found no *CTNNB1* mutation in the drop-out group, and only 5.95% of the control patients had a *CTNNB1* mutation (5/84). These mutations were all missense mutations. We have not isolated any mutations within the *PIK3CA* and *NFE2L2* genes.

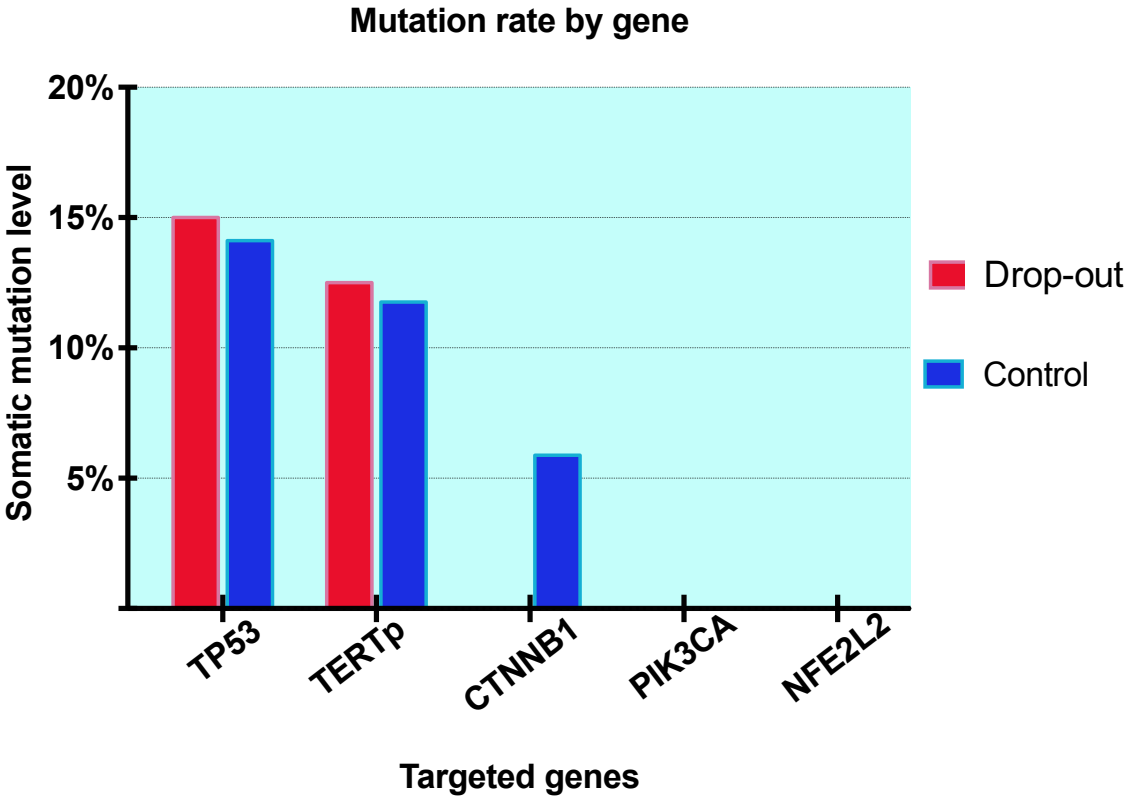

Figure 6: Mutation rate of the targeted genes in the drop-out group compared to control group.

Among patients with detectable ctDNA, 10% of patients had 2 mutations in 2 different genes (*TERTp* and *TP53*) in the drop-out group (1/10), while 22.7% of patients had 2 mutations in 2 different genes in the control group (OR: 0.41 95%CI: 0.05;3.70, p=0.43). Only one patient had a mutation in the *CTNNB1* and *TP53* genes, all other mutations affected *TERTp* and *TP53* genes.

4.5.2.4 Quantification of circulating tumor DNA

In the drop-out from list group, the mean VAF of *TP53* mutations was 2.7% (range: 0.94-7.16%) while it was 1.4% (range: 0.57-4.13%) in the control group. This difference was not statistically significant (p=0.13). For *TERTp* mutations, in the drop-out group it was 0.92% (range: 0.2-2.03%) while it was 0.75% (range: 0.18-2.94%) in the control group (p=0.72) (*Figure 7*). There was no mutation of *CTNNB1* in the drop-out group. In the control group, the mean VAF for *CTNNB1* mutations was 1.7% (range: 0.62-4.92%).

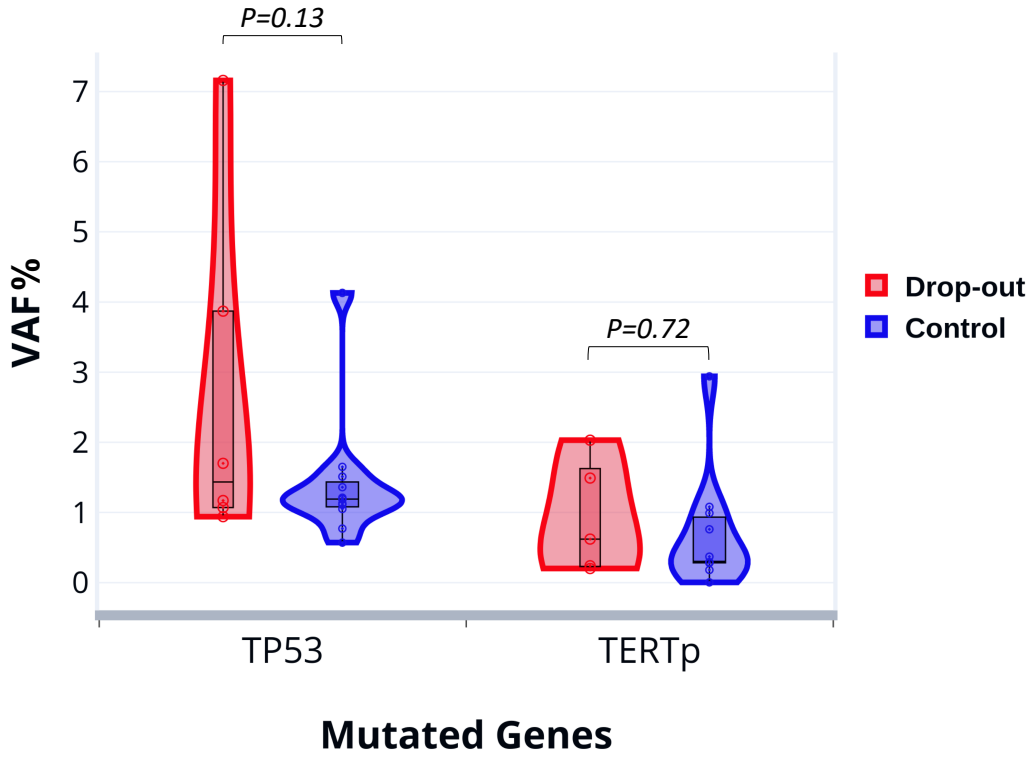

Figure 7: Distribution of VAF according to the mutations of the different target genes in the drop-out group compared to control group.

4.5.3 Correlation between genomics data and clinico-biological characteristics

We investigated whether clinico-biological and imaging features were associated with the following variables: cfDNA concentration, detectability of circulating tumor DNA and somatic mutations and variant allele frequency of mutations. To do this, we carried out these analyses independently of the groups, taking the whole cohort of patients (n=194).

4.5.3.1 cfDNA level

There was no significant correlation between circulating DNA levels and the following clinical factors: age (Spearman p=0.275), number of tumors (Spearman p=0.856), Child Pugh score (Spearman p=0.275), MELD score (Spearman p=0.610), alpha-fœtoprotein level (Spearman p=0.630). However, there was an association between circulating DNA concentration and the size of the largest tumor (Spearman p=0.141); this association was not significant. Regarding etiologies of liver disease, it was for alcohol liver disease that cfDNA rate was most significantly increased (mean of 0.45 ng/µL vs 0.22 ng/µL; p=0.099). This association can be explained by a higher degree of cytolysis, allowing a greater amount of circulating DNA to be released. Finally, there was a clear decreasing trend in circulating DNA levels in patients who had undergone chemoembolization prior to liver transplantation (p=0.077). This association can be explained by the fact that these treatments aim to decrease tumor activity, resulting in a decrease circulating DNA release. The other treatments carried out before listing had no influence on the concentration of circulating DNA (*Table 3).*

			cfDNA concentration (ng/µL) N=194			
		N	**Mean (±SD)**	**Median (IQR)**	**Spearman**	**p value**
Age at listing inscription					0.08	**0.275**
Male gender		176	0.38 (±2.12)	0.11 (0.06;0.22)		**0.687**
Etiology						
HCV Infection	no	140	0.44 (±2.37)	0.11 (0.07;0.24)		**0.205**
	yes	54	0.23 (±0.37)	0.10 (0.04;0.21)		
HBV Infection	no	175	0.39 (±2.13)	0.11 (0.06;0.23)		**0.494**
	yes	19	0.25 (±0.48)	0.09 (0.05;0.21)		
Alcohol	no	59	0.22 (±0.38)	0.09 (0.03;0.21)		**0.099**
	yes	135	0.45 (±2.41)	0.11 (0.07;0.25)		
NASH	no	163	0.40 (±2.20)	0.11 (0.06;0.22)		**0.939**
	yes	31	0.24 (±0.38)	0.11 (0.06;0.33)		
Hémochromatosis	no	186	0.39 (±2.07)	0.11 (0.06;0.22)		**0.944**
	yes	8	0.19 (±0.21)	0.11 (0.06;0.34)		
Child Pugh Class						
A		114	0.27 (±0.43)	0.10 (0.05;0.22)		
B		55	0.22 (±0.26)	0.13 (0.08;0.27)		**0.275**
C		19	0.12 (±0.11)	0.11 (0.04;0.17)		
MELD score					0.04	**0.610**
Number of nodules					- 0.01	**0.856**
Diameter of the largest tumor (mm)					0.11	**0.141**
alpha-fœtoprotein (ng/ml)					0.03	**0.630**
alpha-fœtoprotein (ng/ml)	<100	178	0.39 (±2.11)	0.11 (0.06;0.22)		**0.300**
	≥100	16	0.27 (±0.50)	0.08 (0.00;0.23)		
Treatment before transplantation listing						
Chemoembolization	no	86	0.23 (±0.32)	0.13 (0.08;0.22)		**0.077**
	yes	108	0.49 (±2.70)	0.09 (0.05;0.25)		
Thermoablation	no	153	0.41 (±2.27)	0.10 (0.06;0.25)		**0.995**
	yes	41	0.25 (±0.39)	0.12 (0.06;0.17)		
Hepatic resection	no	174	0.40 (±2.14)	0.11 (0.06;0.22)		**0.647**
	yes	20	0.19 (±0.26)	0.11 (0.06;0.19)		

Table 3: Clinico-biological and imaging factors associated with concentration of cell free DNA in the whole cohort

4.5.3.2 Somatic mutation detection

A total of 196 samples from 194 patients were sequenced using the MiSeq panel Hotspot. In the whole population, at least one mutation in one of the 5 targeted genes could be detected in 48 patients (48/194, 24.7%). No *PIK3CA* or *NFE2L2* mutations were detected in the analysis. No etiology was associated with positivity of circulating tumor DNA. The detectability of ctDNA was not associated with the number of tumors (p=0.504), the maximum size of HCC (p=0.596). Neither the alpha-fœtoprotein level (p=0.954), nor the Child Pugh class (p=0.694), nor the MELD score (p=0.805), was associated with detecting a mutation in the sequencing of circulating DNA (Table 4).

The most common somatic mutations identified were *TP53* in 13.4% (26/194) followed by *TERT* promoter mutations detected in 11.3% (22/194) and *CTNNB1* mutations in 4.1% (8/194).

No etiology of liver disease was associated with the mutation of any specific gene. The Child Pugh or MELD score was also not associated with any somatic mutation. Mutations in the *TP53* gene was associated with the maximum diameter of HCC, but this association was not significant (p=0.142). (Supplementary Data; Table S1). Patients mutated on the *TERT* promoter gene had better prognostic imaging characteristics, without significant association as the maximum size of HCC was 23.5 mm in mutated patients versus 22.5 mm in wild type patients (p=0.322). There was no difference in the number of nodules between *TERTp* mutated and wild-type patients (p=0.567). Alpha-fœtoprotein level was not associated with the presence of *TERT* promoter mutation (p=0.653). For *CTNNB1* mutations, there was a trend towards a higher median level of alpha-fœtoprotein in the mutated group (p=0.32). There was no other association between the presence of a *CTNNB1* mutation and the clinico-biological data of the cohort (Supplementary Data; Table S1).

		Detectabiliy of ctDNA		
		No n=146	**yes** n=48	**P value**
Age at listing inscription				
median (IQR)		60.0 (55;63)	58.0 (53.5;61.5)	**0.599**
Male gender				
n (%)		132 (90.41%)	44 (91.67%)	**0.999**
Etiology				
HCV Infection	yes n(%)	41 (28.08%)	13 (27.08%)	**0.893**
HBV infection	yes n(%)	15 (10.27%)	4 (8.33%)	**0.999**
Alcohol	yes n(%)	101 (69.18%)	34 (70.83%)	**0.829**
NASH	yes n(%)	25 (17.12%)	6 (12.50%)	**0.448**
Hemochromatosis	yes n(%)	4 (2.74%)	4 (8.33%)	**0.106**
Child Pugh Class				
A n(%)		84 (59.15%)	30 (65.22%)	
B n(%)		42 (29.58%)	13 (28.26%)	**0.694**
C n(%)		16 (11.27%)	3 (6.52%)	
MELD score				
median (IQR)		10 (8.0;13.0)	10 (8.0;14.0)	**0.805**
Number of nodules				
median (IQR)		2.0 (1;3)	1.00 (1;3)	**0.504**
Diameter of the largest tumor (mm)				
median (IQR)		23.0 (16.0;32.0)	23.5 (19.0;36.5)	**0.596**
alpha-fœtoprotein (ng/ml)				
median (IQR)		7.3 (3.9;30.3)	8.6 (4.0;15.6)	**0.954**
alpha-fœtoprotein (ng/ml)				
<100 n(%)		134 (91.78%)	44 (91.67%)	**0.999**
≥100 n(%)		12 (8.22%)	4 (8.33%)	
Treatment before transplantation listing				
Chemoembolization	yes n(%)	82 (56.16%)	26 (54.17%)	**0.809**
Thermoablation	yes n(%)	32 (21.92%)	9 (18.75%)	**0.641**
Hepatic resection	yes n(%)	15 (10.27%)	5 (10.42%)	**0.999**

Table 4: Clinico-biological and imaging factors associated with ctDNA detectability in the whole cohort.

4.5.3.3 Variant Allele frequency (VAF)

Finally, we investigated whether there was an association between the level of circulating tumor DNA and clinico-biological characteristics of our population. The amount of ctDNA is defined by the VAF, i.e. the proportion of alleles containing the targeted mutation in the whole circulating DNA.

For TP53 mutations, there was an inverse correlation between the size of the largest tumor and the VAF of the mutations (Spearman's rank coefficient: -0.35) This association was not significant (p=0.079). No other clinical-biological parameters were associated with the TP53 mutations VAF. Analysis of the TERTp mutation VAF showed a statistically significant association between the number of HCC and the VAF (Spearman's rank coefficient: 0.45; p=0.035). Similarly, alpha-fœtoprotein levels appeared to be higher in patients with higher TERTp mutation VAF (Spearman p=0.098). Interestingly, patients who underwent chemoembolization treatment had a lower TERTp mutation VAF (p=0.056), suggesting the possibility of monitoring treatment response by ctDNA levels. This result was not validated with other treatments. Finally, the NASH incidence seemed significantly higher in patients with a stronger *TERTp* mutation VAF (p=0.06). The VAF of *CTNNB1* mutations was associated with age (p=0.014). There was a trend between the amount of *CTNNB1* mutated allele and the alpha-fœtoprotein level (p=0.127) and the diameter of the largest tumor (p=0.168) (*Table 5*).

		TP53 VAF N=26			TERTp VAF N=22			CTNNB1 VAF N=8		
		N		p-value	N		p-value	N		p-value
Age at listing inscription										
Spearman's rank			0.28	0.162		0.21	0.337		0.81	**0.014**
Male Gender										
median (IQR)		23	1.19 (1.05;1.70)	0.936	20	0.56 (0.29;1.29)	0.530	8	1.21 (0.83;2.91)	
Etiology										
HCV Infection	yes median (IQR)	7	1.19 (1.13;1.71)	0.664	6	0.54 (0.28;0.76)	0.854	2	2.77 (0.62;4.92)	0.999
HBV Infection	yes median (IQR)	2	1.09 (1.07;1.11)	0.336	1	0.99 (0.99;0.99)	0.478	1	1.43 (1.43;1.43)	0.513
Alcohol	yes median (IQR)	19	1.21 (1.05;1.70)	0.418	16	0.36 (0.26;0.92)	0.083	6	1.21 (1.01;1.43)	0.739
NASH	yes median (IQR)	3	1.51 (1.18;4.13)	0.244	4	1.56 (0.85;2.03)	0.061	0	0 (0;0)	
Hémochromatosis	yes median (IQR)	2	1.76 (0.97;2.55)	1.000	2	1.69 (0.71;2.67)	0.170	1	4.39 (4.39;4.39)	0.275
Child Pugh Class										
A median (IQR)		19	1.19 (1.05;1.70)		13	0.76 (0.29;1.49)		6	1.23 (1.01;4.39)	
B median (IQR)		6	1.32 (0.77;1.71)	0.709	5	0.37 (0.29;0.42)	0.520	2	1.02 (0.65;1.39)	0.505
C median (IQR)		1	1.07 (1.07;1.07)		2	0.60 (0.49;0.70)		0	0 (0;0)	
MELD score										
Spearman's rank			0.16	0.460		-0.31	0.215		-0.17	0.690
Number of nodules										
Spearman's rank			0.10	0.624		0.45	**0.035**		0.22	0.604
Diameter of the largest tumor (mm)										
Spearman's rank			-0.35	0.079		0.003	0.987		0.54	0.168
alpha-fœtoprotein (ng/ml)										
Spearman's rank			-0.09	0.663		0.12	0.595		0.05	0.911
alpha-fœtoprotein (ng/ml)										
<100 median (IQR)		23	1.19 (1.05;1.70)	0.968	21	0.49 (0.29;0.99)	0.098	7	1.04 (0.65;1.43)	0.127
≥100 median (IQR)		3	1.17 (1.07;2.55)		1	2.94 (2.94;2.94)		1	4.92 (4.92;4.92)	
Treatment before transplantation listing										
Chemoembolization	yes median (IQR)	15	1.19 (1.13;1.70)	0.436	10	0.33 (0.28;0.70)	0.056	4	1.02 (0.81;1.23)	0.248
Thermoablation	yes median (IQR)	6	1.35 (0.94;1.70)	0.927	4	0.69 (0.41;0.88)	0.966	0	0 (0;0)	
Hepatic resection	yes median (IQR)	3	1.51 (0.74;4.13)	0.718	3	0.99 (0.71;1.08)	0.231	0	0 (0;0)	

Table 5: Correlation between the proportion of mutated alleles (VAF) and the clinico-biological and imaging characteristics of the whole population.

4.6 Discussion

In this study we report the results of circulating tumor DNA analysis in a cohort of patients listed for liver transplantation for HCC. We evaluated the performance of ctDNA to predict the risk of recurrence or drop out of the liver transplant list. The results did not allow us to prove the predictive quality of detectability or quantification of ctDNA levels in HCC transplant patients. However, we were able to demonstrate correlations between ctDNA levels and clinico-biological markers, positioning ctDNA as a potential biomarker.

Analysis of ctDNA has already demonstrated its interest in determining molecular characteristics of a tumor or in monitoring its evolution according to therapeutics (45). Measurement of ctDNA can also specifies the prognosis of a tumor (46), and detectability of ctDNA is highly correlated with the tumor burden of the disease (37). In our work, we proved that ctDNA is detectable in HCC patients at the time of listing for liver transplantation with an early stage of the disease. First, we have shown that it is possible to screen the mutations involved in the tumorigenesis of HCC. Nearly 25% of patients in the cohort have at least one somatic mutation in the genes targeted by our panel. In addition, we have shown interesting correlations between *TERTp* mutation VAF and the number of HCC nodules, the level of alpha-fœtoprotein and the etiology of the associated liver disease. A previous work has found that *TERTp* mutation was a predictive factor of progression disease in HCC treated by surgery (47). The close connection between ctDNA and tumor features makes ctDNA an ideal potential tumor marker for recurrence.

No studies have investigated the evaluation of ctDNA in patients considered for liver transplantation for HCC. These patients have the particularity of having a better prognosis tumor since they are highly selected with Milan criteria, which are based on tumor size and number and with a long waiting time offering a selection for fast progression. In our study, all patients were initially eligible for liver transplantation, so they were only tumors that were considered to be good prognosis. This contributes to the difficulty of this work, since the difference between the two groups is very moderate. A lack of power in relation to small number of events also contributed to the non-significant results. Today, several biomarkers can predict the risk of liver transplant recurrence, such as alpha-fœtoprotein level, size and number of nodule, downstaging treatment, profile of microvascular invasion, tumor differentiation grade (48–52). On a molecular side, other biomarkers provide tumor prognosis, some are poor prognostic such as the presence of *TP53* mutations, absence of *CTNNB1* mutation, progenitor cell markers (i.e., CK19 signature), fractional allelic imbalance, others are good prognostic such as expression of miR-199a-5p, miR-122, miR-126 8a (53–57). But they all require tissue analysis. Therefore, there is an urgent need to define sensitive and accessible biomarkers to refine HCC prognosis.

It should be noted that ctDNA, is composed of fragments of a hundred nucleotides only and is extremely delicate and reactive to environmental parameters.

The average lifetime of nucleotides in blood is between 15 minutes and two hours (58). As a matter of fact, ctDNA is detected better in plasma than in serum because allele frequency is significantly higher in plasma. In serum, genomic DNA of non-cancerous origin is predominant (59). Indeed, within the serum, the lysis of white blood cells, especially neutrophils polynuclear cells, is more pronounced, especially at room temperature, which explains the predominance of non-tumor genomic DNA. In our work, all samples were derived from plasma. Others parameters are involved in measuring the level of circulating DNA, such as the collection tube used, the time between sampling and plasma recovery, the time between sampling and freezing, the storage temperature, or the sample age (60). Our samples are 6 to 10 years old, which could also affect their purity. Limited data are available concerning blood draw procedure and pre-analytical variables that influence degradation of cfDNA or contamination by cellular genomic DNA derived from leukocyte lysis (61). Likewise, changes in cfDNA purification methods and various protocol modifications may affect the yield and purity of cfDNA.

ctDNA is quantified by measuring the rate of mutated alleles of interest genes in the whole cell free DNA. This is called Variant Allele Frequency (VAF). Generally, the rate of mutated circulating DNA is minimal compared to the overall germinal cfDNA rate. It is often in the order of a hundredth or even a thousandth (62). This is why it requires high-performance sequencing tools. In our study, this rate is consistent with data from literature as when ctDNA was detectable its mutated allele rate was between 0.18% and 7.16%. This rate is also dependent on the amplification coverage. This was largely satisfying in our work since it was between 5000 to 40000x per amplicons in *TP53*, *TERTp* and *CTNNB1* genes. Despite this high coverage, ctDNA was detectable in only 25% of the samples. This rate is lower than that of other published series, where ctDNA detectability rates are rather between 30% and 80%, depending on the targeted genes (36,63,64). Several factors can be assumed to explain the low detectability of ctDNA. One of the first is the tumor load of the patients included in our study. Indeed, all patients were included for liver transplantation, and therefore were considered to have low tumor volume. The majority of patients had even benefited treatment prior to waiting list inscription such as chemoembolization, thermoablation or surgical resection. It is widely recognized that detectability of ctDNA is correlated with tumor load (65).

The gene panel used to sequence the circulating plasma DNA targeted only 5 genes, and only hotspots genes, i.e. regions of the gene that are known to be most frequently mutated. It is recognized that more than 75% of HCCs have at least, a mutation of *TERTp*, *CTNNB1* or *TP53*(14). However, hepatocellular carcinoma is a very heterogeneous tumor caused by many different genetic and epigenetic processes. Some genes often involved in hepatocarcinogenesis, such as *AXIN1*, *ARID1A*, *ARID2*, *RPS6KA3*, *CDKN2A*, *RB1*, JAK1 are not targeted by our panel. By adding more relevant hotspots, the detection rate could be furtherly improved, amplifying the advantages of ctDNA. In our study, we detect a higher proportion of

TP53 mutations (13%) than *TERTp* (11%), which is surprising given the data in the HCC genomics literature(66). Indeed, *TERTp* mutation is the most common point mutation in HCC, and is also found in preneoplastic or dysplastic nodules. But its sequencing conditions are often difficult due to the importance of the GC rich regions, which are hardly denaturable. Sanger sequencing remains one of the safest methods to sequence this region of the genome. Other work on ctDNA in HCC has found higher mutation rates in *TP53* than in *TERTp* (46). In our study we found no evidence of *PIK3CA* or *NFE2L2* mutations, which are known to be involved in liver carcinogenesis, particularly in the response to oxidative stress (*NFE2L2*). This is mostly explained by the lack of coverage obtained for these amplicons during the amplification stages. This could be related to poor quality of primers and restricted targeting to the hotspots (i.e. 4 amplicons on exon 2 of *NFE2L2* and 6 amplicons on exons 10 and 21 of the *PIK3CA* gene).

There are several methods for ctDNA analysis: those based on the detection of a small number of targeted variants such as PCR methods or those that focus on a broader coverage such as NGS method (60). In our study we used NGS methods with Illumina system. It is a qualitative and quantitative method that allows detection of mutations, deletion/amplifications, gene translocations, and fusion transcripts. It allows quantification of relative allele frequency, which is very vulnerable to PCR amplifications bias. Many other studies on ctDNA in oncology have used PCR in microdroplets also called digital PCR (dPCR). PCR and NGS methods cannot achieve the same results because of different assay performance characteristics, detection limits and different targets of the genome. Unfortunately, today none of these methods are formally recommended, or proven to be superior. In the work of Labgaa.I et al (67) they compared dPCR and NGS methods to analyze cfDNA of 8 patients. They compared concordance of sequencing results between tissue and plasma and found concordance with NGS methods for 9 out of 21 (43%) mutations, on targeted genes such as *TERT* promoter, *TP53*, *CTNNB1*, *JAK1* or *AXIN1*. These 9 displayed high VAF. Moreover, they analyzed these mutations by dPCR and found consistent results with NGS, with VAF between 0.3% and 2.12%. dPCR has the advantage of an extensive amplification, but the targeted genes must be known and their amount must be restricted. This makes the method interesting in the setting of tumors with few genes involved, but in HCC it is necessary to focus on sequencing coverage taking into account the heterogeneity of the carcinogenesis phenomena involved. That is why we choose NGS method. It allows the simultaneous assessment and detection of multiple genetic aberrations and copy number changes, including targeted techniques.

Another difficulty encountered with the measurement of tumor burden by ctDNA is the variability of each tumor for ctDNA release. For example, it is proved that ctDNA levels are more often detectable in tumors of the bladder, colon, stomach or ovary and much less detectable in cancers of the kidney, the prostate, the thyroid or the central nervous system (CNS) (65). In tumors of CNS, the detection of ctDNA

is almost zero because nucleic acids do not pass the blood-brain barrier. In the context of these tumors, ctDNA research will be carried out preferentially in other biological fluids such as CSF (68). For HCC, it would be interesting to perform the same analysis in ascites to diagnose early HCC. Additionally, several pathological conditions other than cancer can alter the amount of cfDNA especially by increasing it. This is the case of tissue stress, such as tissue injury, exercise or inflammatory processes (69). Very low frequency tumor-related mutations have even been observed in healthy patients or smokers, confirming that ctDNA is not necessarily cancer-specific (70). The analysis of ctDNA still requires development, standardization and further work will be necessary to improve the technical performance of this tool.

The primary limitation of our study is the lack of analysis of associated biopsies. Indeed, it has been shown that characterizing genetic profile of a tumor by using cfDNA sequencing is possible. However, today, while ctDNA analysis remains in the experimental field, it is necessary to confirm and validate the detection of genetic variants by analysis of the concomitant tissue or peripheral blood mononuclear cells (PBMC). As shown by Ng C.K.Y et al (63) showed that ctDNA was detectable in 63% of HCC patients but, this rate was reduced to 27% when no concomitant biopsy was available, which corresponds to the values obtained in our study. The concomitant biopsy helps on the one hand to validate the detected mutation and on the other hand to guide the mutation screening. Howell J. et al determined a sensitivity of 30% but a specificity of 100% for the diagnosis of HCC by ctDNA (71). Actually, it is shown that false-positive plasma genotyping could be related to clonal hematopoiesis with non-malignant mutations which are harbored by hematopoietic cells (72). So mutations detected in plasma could be related to clonal hematopoiesis, and plasma genotyping should be paired with PBMC sequencing to not misdiagnose malignancy.

In our study, seven samples had an available tumor tissue at the time of liver transplantation already sequenced in the laboratory. Two patients had no mutations either the sequenced tumor or in the plasmatic DNA. One patient had a *TERTp* mutation in the ctDNA that was found in the tumor. And four patients had a *TERTp* mutation that was not detected in the ctDNA. The profiling of these tumors was performed by Sanger sequencing. There were no *TP53* or *CTNNB1* mutations detected. The delay between sampling in plasma and tumor was between 62 to 835 days. During this waiting time, HCC treatments such as chemoembolization, radiofrequency ablation may have affected tumor burden which could explain these discrepancies.

The advantage of plasma DNA analysis is its availability, accessibility and the non-invasiveness of its sampling. This makes it easy to regularly monitor the evolution of the disease, by measuring ctDNA rates in response to therapeutics. It is an informational and dynamic biomarker. In our study, we found a VAF of *TERTp* mutations decreased in patients who received pre-collection chemoembolization ($p<0.1$). Many works report an evolution of the targeted mutations VAF, notably a

decrease when there is a response to treatment (25,73,74). As it has been shown, *TP53*, *TERTp* or *CTNNB1* mutation detection in plasma after surgical treatment, is associated with a decrease of recurrence-free survival (26). In our work, it would have been interesting to sequence the plasma DNA at several time points during the waiting time in order to evaluate more precisely the response to treatment. Indeed, some patients included in our study benefited from waiting treatments such as chemoembolization, thermal ablation or resection. These one most probably influenced the variation in ctDNA levels by certainly reducing their detectability. Patients with positive and detectable VAF after treatment, and therefore reported as ctDNA-positive, belong to the residual microscopic disease group. These patients are considered to be at high risk of post-transplant recurrence. Therefore, ctDNA could be a more sensitive biomarker than the AFP assay, which lacks precision and could be complementary to the radiological evolution according to mRECIST criteria.

Another challenge with use of ctDNA would be the prediction of treatment response based on tumor mutation profiles. In the age of personalized medicine, many cancers are treated in the context of the molecular characteristics of the tumors. This is the case, for example, in colorectal cancer, where the *RAS* status predicts response to anti-EGFR treatment, in lung cancer where the presence of an *EGFR* activating mutation provides access to treatment with tyrosine kinase inhibitors, and in melanoma BRAF mutation predicts the response to BRAF inhibitors. Thus, as shown by Siravegna et al (75), monitoring tumor clone composition with ctDNA allows efficient and simple monitoring of tumor evolution, and detection of the appearance of secondary resistance. However, in HCC there is a major accumulation of genetic abnormalities, with no real "driver" abnormality that is currently frequent and targetable. The molecular evolution of a tumor often reveals significant tumor heterogeneity with the appearance of subclones whose molecular profiles are different from the initial characteristics and which evolve for their own in a parallel process. This tumor heterogeneity does not ensure the representability of a sample obtained by biopsy. To demonstrate this molecular evolution, in clinical practice it is necessary to biopsy the patient several times, which is inconceivable. In our study, we demonstrated the role of ctDNA, as a liquid biopsy which can detect very accurately strong somatic mutations within the tumor, disregarding intra-tumor heterogeneity. Some plasma even had 2 or even 3 somatic mutations concomitantly. Huang A. et al (36) showed that ctDNA analysis could detect 70% of the total mutations in a multi-site biopsy. Thus, ctDNA analysis is a very useful tool which is already being used in clinical practice, particularly in thoracic oncology. Nevertheless, in HCC, there is a lack of druggable mutations and no robust marker is so far predictive of response to TKI treatment or immunotherapy. Some retrospective works are looking at the prediction of response to TKI inhibitors in HCC, such as the amplification of VEGFA or FGF3/4 would predict the response to sorafenib (76) or serum levels of LAP TGF-b1, MIP-1a, LOX-1 and ANG-1 would predict the response to regorafenib (77). Individualizing HCC therapy should remain a priority and these works need to be further studied in order to determine their place in the clinical field.

4.7 Conclusion

In our work we were not able to identify a prognostic role of circulating tumor DNA in order to help identifying patients at risk of tumor recurrence after liver transplantation nor patients at risk of drop-out from the waiting list for tumor progression. Similarly, we have not demonstrated any correlation between the detectability of ctDNA, or the presence of a somatic mutation in the targeted genes and the clinical-biological features. More studies seem necessary to standardize techniques and develop our analysis protocols of ctDNA in order to validate its contribution both in the diagnostic and prognostic fields.

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4.9 Supplementary Data

	TP53			TERTp			CTNNB1		
	Wild Type N=168	Mutated N=26	P value	Wild type N=172	Mutated N=22	P value	Wild Type N=186	Mutated N=8	P value
Age at listing inscription									
median (IQR)	59.0 (54;63)	58.50 (54.00;61.00)	**0.904**	60.0 (55;63)	57.5 (52;61)	**0.212**	59.0 (54;63)	61.5 (57.5;63)	**0.205**
Male gender									
n (%)	153 (91.07%)	23 (88.46%)	**0.715**	156 (90.70%)	20 (90.91%)	**1.000**	168 (90.32%)	8 (100.00%)	**1.000**
Etiology									
HCV Infection	47 (27.98%)	7 (26.92%)	**0.911**	48 (27.91%)	6 (27.27%)	**0.950**	52 (27.96%)	2 (25.00%)	**1.000**
HBV infection	17 (10.12%)	2 (7.69%)	**1.000**	18 (10.47%)	1 (4.55%)	**0.702**	18 (9.68%)	1 (12.50%)	**0.569**
Alcohol	116 (69.05%)	19 (73.08%)	**0.677**	119 (69.19%)	16 (72.73%)	**0.734**	129 (69.35%)	6 (75.00%)	**1.000**
NASH	28 (16.67%)	3 (11.54%)	**0.773**	27 (15.70%)	4 (18.18%)	**0.759**	31 (16.67%)	0 (0.00%)	**0.360**
Hemochromatosis	6 (3.57%)	2 (7.69%)	**0.292**	6 (3.49%)	2 (9.09%)	**0.226**	7 (3.76%)	1 (12.50%)	**0.291**
Child Pugh Class									
A n(%)	95 (58.64%)	19 (73.08%)		101 (60.12%)	13 (65.00%)		108 (60.00%)	6 (75.00%)	
B n(%)	49 (30.25%)	6 (23.08%)	**0.367**	50 (29.76%)	5 (25.00%)	**0.938**	53 (29.44%)	2 (25.00%)	**0.876**
C n(%)	18 (11.11%)	1 (3.85%)		17 (10.12%)	2 (10.00%)		19 (10.56%)	0 (0.00%)	
MELD score									
median (IQR)	10 (8.0;13.0)	9 (7.5;11.0)	**0.223**	10 (8.0;13.0)	10.5 (9.0;15.0)	**0.357**	10 (8.0;13.0)	9 (8.0;12.0)	**0.487**
Number of nodules									
median (IQR)	2.0 (1;3)	1.5 (1;3)	**0.621**	2.0 (1;3)	1.0 (1;3)	**0.567**	2.0 (1;3)	2.0 (1;3)	**0.795**
Diameter of the largest tumor (mm)									
median (IQR)	23.0 (16.0;30.0)	25.5 (20.0;40.0)	**0.142**	23.5 (16.0;33.0)	22.5 (15.0;26.0)	**0.322**	23.0 (16.0;32.0)	24.5 (21.0;33.5)	**0.565**
alpha-fœtoprotein (ng/ml)									
median (IQR)	7.7 (4.0;28.0)	8.6 (4.0;14.2)	**0.747**	7.8 (4.0;23.0)	7.3 (4.0;19.0)	**0.653**	7.5 (4.0;21.0)	12.3 (6.0;37.7)	**0.322**
alpha-fœtoprotein (ng/ml)									
<100 n(%)	155 (92.26%)	23 (88.46%)	**0.455**	157 (91.28%)	21 (95.45%)	**1.000**	171 (91.94%)	7 (87.50%)	**0.504**
≥100 n(%)	13 (7.74%)	3 (11.54%)		15 (8.72%)	1 (4.55%)		15 (8.06%)	1 (12.50%)	
Treatment before transplantation listing									
Chemoembolization	93 (55.36%)	15 (57.69%)	**0.823**	98 (56.98%)	10 (45.45%)	**0.306**	104 (55.91%)	4 (50.00%)	**0.735**
Thermoablation	35 (20.83%)	6 (23.08%)	**0.794**	37 (21.51%)	4 (18.18%)	**1.000**	41 (22.04%)	0 (0.00%)	**0.207**
Hepatic resection	17 (10.12%)	3 (11.54%)	**0.736**	17 (9.88%)	3 (13.64%)	**0.707**	20 (10.75%)	0 (0.00%)	**1.000**

Table S1: Clinico-biological and imaging factors associated with somatic mutations detection in the whole cohort.

5. CONCLUSION

Thèse soutenue par : Olivier MSIKA

Titre : Apport de l'ADN tumoral circulant comme marqueur pronostique post-transplantation hépatique pour carcinome hépatocellulaire : une étude prospective multicentrique.

Le carcinome hépatocellulaire est la seule tumeur solide pouvant être traitée par la transplantation. Celle-ci permet une survie à 5 ans proche de 80%. La principale cause de mortalité post-greffe est représentée par la récidive tumorale. A l'heure du développement de la génomique, et de son apport dans la médecine personnalisée, nous avons cherché à démontrer le rôle de l'ADN tumoral circulant comme marqueur prédictif de récidive et de sortie de liste de transplantation pour progression tumorale. L'ADN tumoral circulant est un marqueur biologique d'intérêt car il apporte une information quantitative et qualitative. Il permet de décrire le profil génétique d'une tumeur, en contenant les mutations somatiques impliquées dans la carcinogenèse. Il a donc la qualité d'un outil diagnostic. Et il permet de déterminer la charge tumorale en reflétant le taux de mutations, ce qui en fait un outil pronostique. Peu de marqueurs sont aussi informatifs dans le domaine du carcinome hépatocellulaire. Dans notre étude, nous avons prouvé que l'ADN tumoral circulant est un biomarqueur précis, dont le taux est corrélé à plusieurs données clinico-biologiques ou radiologiques (taille de la plus grosse tumeur, nombre de tumeur, étiologie…) et reflète la charge tumorale. Cependant nous n'avons pas montré qu'il permettait de prédire le pronostic post-transplantation hépatique des patients greffés pour carcinome hépatocellulaire.

Ce travail à mi-chemin entre l'expérimental et la clinique, est un premier pas dans le domaine de la transplantation hépatique. Il est nécessaire de mieux caractériser ces tumeurs très hétérogènes et d'individualiser la prise en charge des patients et leurs maladies. Pour cela, d'autres études semblent nécessaires afin de standardiser les méthodes d'analyse de l'ADN tumoral circulant, développer des panels de gènes plus larges et plus précis et affiner les processus d'analyses bio-informatiques.

VU ET PERMIS D'IMPRIMER
Grenoble, le 4/9/2020

LE DOYEN
Pr. Patrice Morand

LE PRESIDENT DU JURY
Pr. Thomas DECAENS

Pour le Président
et par délégation
Le Doyen de Médecine
Pr. Patrice MORAND

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Merci au Professeur Bruno Bonaz, pour votre gentillesse. Merci de nous avoir transmis votre passion pour la médecine et la clinique.

Aux chefs et assistants qui ont eu la patience de m'encadrer Camille (je suis ravi d'être ton futur collègue), Laetitia, Virginie, Romain, Justine (pour ton zèle exemplaire), Thomas (pour ta disponibilité et ton aide toujours précieuse) Bleuenn, et enfin le Professeur Gaël et son aisance dans toute situation!

A toutes les équipes infirmièr(e) et aide-soignant(e) du 7ème B, 7ème C, CIRE. Aux secrétaires médicales dont l'aide est précieuse. A Sylvie Dorne qui pense toujours à moi lorsqu'il y a quelque part, quelque chose à manger …

A l'équipe médicale d'oncologie de l'Institut Daniel Hollard qui m'ont encadré en 5ème semestre et que je vais rejoindre avec grand plaisir.

Merci à l'équipe d'oncologie digestive du Centre Léon Berard, Françoise Desseigne, Christelle de La Fouchardière, Matthieu Sarabi, Clémence Morin, qui m'ont accueilli en inter-CHU et qui nous ont énormément appris. Vous m'avez transmis l'intérêt que je porte aujourd'hui pour l'oncologie digestive. Je garderai un très bon souvenir de votre équipe et de ce stage et j'espère avoir l'occasion de collaborer avec vous.

A mes co-internes passés et actuels, Gael, Justine, Sandie, Mélodie, Aude (merci de toujours m'accueillir en endoscopie), Thomas L, Laurence, Bleuenn, Dysmas, Marion, Baptiste (pour cette session aventureuse, voire périlleuse à la Bastille !), Laurine, Thomas C, Laetitia & Sabine (mes acolytes de Chambéry), Sylvain, Sophie, Marion, Victoria, Alia, Pauline, Fabien et les plus jeunes qui m'ont supporté en avant-dernier semestre Mathilde, Clara, Justine et Léa.

A Olivier, le plus stressant des compagnons de thèse ! Mais fidèle compagnon de papotage !
A Loïc, avec qui j'ai fait mes premiers pas dans l'internat, pour sa passion pour l'OM, sa présence, son soutien au quotidien en premier semestre puis dans cette magnifique collocation et surtout pour son attitude toujours aussi décontracté et insouciante…
Mention spéciale à Théo (ma très grande gueule), co-interne mais pas moins ami. Ton enjouement constant est un exemple. Pour toutes ces litres des bières bus pendant 5 ans, et les autres litres de bières que nous boirons les prochaines années. Merci d'avance pour tous les avis hépato que tu vas me donner à l'avenir !

A tous mes fidèles et loyaux amis parisiens : Mathieu, Mazyar, Cyprien, Baptiste, Christophe, Kévin, qui, malgré les kilomètres restent mes amis de toujours.

A ma Famille,
D'abord ma mère, merci d'avoir toujours cru en moi, en nous. Merci de t'être sacrifiée pour tirer de nous le meilleur. Mon éducation n'a pas due être tous les jours facile, surtout au sein d'une fratrie de quatre garçons, mais aujourd'hui je ne peux que te

remercier de t'être battue pour notre éducation, car si j'en suis là c'est en grande partie grâce à toi.

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A mon Amour, Laura. Je t'aime.
Tu es le pilier de ma vie, les mots sont insuffisants pour te remercier. Pas un jour ne passe sans que je n'estime la chance d'avoir croisé ton chemin. Partager ma vie avec toi me rend heureux. Merci pour ton soutien infaillible, je sais que je peux compter sur toi.

A mon grand-père, Paul, pour son amour.

7. LISTE DES PROFESSEURS ET MAITRES DE CONFERENCE

UFR de Médecine
UNIVERSITÉ Grenoble Alpes

Doyen de la Faculté : **Pr. Patrice MORAND**

Année 2019-2020

ENSEIGNANTS DE L'UFR DE MEDECINE

CORPS	NOM-PRENOM	Discipline universitaire
PU-PH	ALBALADEJO Pierre	Anesthésiologie-réanimation et médecine péri-opératoire
PU-PH	APTEL Florent	Ophtalmologie
PU-PH	ARVIEUX-BARTHELEMY Catherine	Chirurgie viscérale et digestive
PU-PH	BAILLET Athan	Rhumatologie
PU-PH	BARONE-ROCHETTE Gilles	Cardiologie
PU-PH	BAYAT Sam	Physiologie
MCF Ass.MG	BENDAMENE Farouk	Médecine Générale
PU-PH	BENHAMOU Pierre Yves	Endocrinologie, diabète et maladies métaboliques
PU-PH	BERGER François	Biologie cellulaire
MCU-PH	BIDART-COUTTON Marie	Biologie cellulaire
PU-PH	BLAISE Sophie	Chirurgie vasculaire ; médecine vasculaire
MCU-PH	BOISSET Sandrine	Bactériologie-virologie
PU-PH	BOLLA Michel	Cancérologie-Radiothérapie
PU-PH	BONAZ Bruno	Gastroentérologie, hépatologie, addictologie
PU-PH	BONNETERRE Vincent	Médecine et santé au travail
PU-PH	BOREL Anne-Laure	Nutrition
PU-PH	BOSSON Jean-Luc	Biostatistiques, informatique médicale et technologies de communication
MCU-PH	BOTTARI Serge	Biologie cellulaire
PR Ass.MG	BOUCHAUD Jacques	Médecine Générale
PU-PH	BOUGEROL Thierry	Psychiatrie d'adultes
PU-PH	BOUILLET Laurence	Médecine interne
MCU-PH	BOUSSAT Bastien	Epidémiologie, économie de la santé et prévention
PU-PH	BOUZAT Pierre	Anesthésiologie-réanimation et médecine péri-opératoire
PU-PH	BRAMBILLA Christian	Pneumologie
PU-PH	BRAMBILLA Elisabeth	Anatomie et cytologie pathologiques
MCU-PH	BRENIER-PINCHART Marie Pierre	Parasitologie et mycologie
PU-PH	BRICAULT Ivan	Radiologie et imagerie médicale
PU-PH	BRICHON Pierre-Yves	Chirurgie thoracique et cardiovasculaire
MCU-PH	BRIOT Raphaël	Thérapeutique-médecine de la douleur
MCU-PH	BROUILLET Sophie	Biologie et médecine du développement et de la reproduction
PU-PH	CAHN Jean-Yves	Hématologie
PU-PH	CARPENTIER Patrick	Chirurgie vasculaire, médecine vasculaire
PR Ass.MG	CARRILLO Yannick	Médecine Générale
PU-PH	CESBRON Jean-Yves	Immunologie
PU-PH	CHABARDES Stephan	Neurochirurgie
PU-PH	CHABRE Olivier	Endocrinologie, diabète et maladies métaboliques
PU-PH	CHAFFANJON Philippe	Anatomie

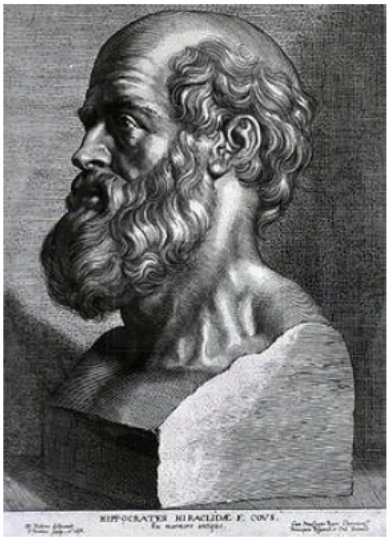
CORPS	NOM-PRENOM	Discipline universitaire
PU-PH	CHARLES Julie	Dermato-vénéréologie
MCF Ass.MG	CHAUVET Marion	Médecine Générale
PU-PH	CHAVANON Olivier	Chirurgie thoracique et cardio- vasculaire
PU-PH	CHIQUET Christophe	Ophtalmologie
PU-PH	CHIRICA Mircea	Chirurgie viscérale et digestive
PU-PH	CINQUIN Philippe	Biostatistiques, informatique médicale et technologies de communication
MCU-PH	CLAVARINO Giovanna	Immunologie
PU-PH	COHEN Olivier	Histologie, embryologie et cytogénétique
PU-PH	COURVOISIER Aurélien	Chirurgie infantile
PU-PH	COUTTON Charles	Génétique
PU-PH	COUTURIER Pascal	Gériatrie et biologie du vieillissement
PU-PH	CRACOWSKI Jean-Luc	Pharmacologie fondamentale, pharmacologie clinique
PU-PH	CURE Hervé	Cancérologie
PU-PH	DEBATY Guillaume	Médecine d'Urgence
PU-PH	DEBILLON Thierry	Pédiatrie
PU-PH	DECAENS Thomas	Gastro-entérologie, Hépatologie
PU-PH	DEMATTEIS Maurice	Addictologie
PU-PH	DEMONGEOT Jacques	Biostatistiques, informatique médicale et technologies de communication
MCU-PH	DERANSART Colin	Physiologie
PU-PH	DESCOTES Jean-Luc	Urologie
PU-PH	DETANTE Olivier	Neurologie
MCU-PH	DIETERICH Klaus	Génétique
MCU-PH	DOUTRELEAU Stéphane	Physiologie
MCU-PH	DUMESTRE-PERARD Chantal	Immunologie
PU-PH	EPAULARD Olivier	Maladies infectieuses ; Maladies tropicales
PU-PH	ESTEVE François	Biophysique et médecine nucléaire
MCU-PH	EYSSERIC Hélène	Médecine légale et droit de la santé
PU-PH	FAUCHERON Jean-Luc	Chirurgie viscérale et digestive
MCU-PH	FAURE Julien	Biochimie et biologie moléculaire
PU-PH	FERRETTI Gilbert	Radiologie et imagerie médicale
PU-PH	FEUERSTEIN Claude	Physiologie
PU-PH	FONTAINE Éric	Nutrition
PU-PH	FRANCOIS Patrice	Epidémiologie, économie de la santé et prévention
MCU-MG	GABOREAU Yoann	Médecine Générale
PU-PH	GARBAN Frédéric	Hématologie ; Transfusion
PU-PH	GAUDIN Philippe	Rhumatologie
PU-PH	GAVAZZI Gaétan	Gériatrie et biologie du vieillissement
PU-PH	GAY Emmanuel	Neurochirurgie
MCU-PH	GILLOIS Pierre	Biostatistiques, informatique médicale et technologies de communication
PU-PH	GIOT Jean-Philippe	Chirurgie plastique, reconstructrice et esthétique
MCU-PH	GRAND Sylvie	Radiologie et imagerie médicale
PU-PH	GRIFFET Jacques	Chirurgie infantile
MCU-PH	GUZUN Rita	Nutrition
PU-PH	HAINAUT Pierre	Biochimie et biologie moléculaire
PU-PH	HALIMI Serge	Nutrition
PU-PH	HENNEBICQ Sylviane	Biologie et médecine du développement et de la reproduction
PU-PH	HOFFMANN Pascale	Gynécologie-obstétrique

CORPS	NOM-PRENOM	Discipline universitaire
PU-PH	HOMMEL Marc	Neurologie
PU-MG	IMBERT Patrick	Médecine Générale
PU-PH	JOUK Pierre-Simon	Génétique
PU-PH	KAHANE Philippe	Physiologie
MCU-PH	KASTLER Adrian	Radiologie et imagerie médicale
PU-PH	KRAINIK Alexandre	Radiologie et imagerie médicale
PU-PH	LABARERE José	Epidémiologie, économie de la santé et prévention
MCU-PH	LABLANCHE Sandrine	Endocrinologie, diabète et maladies métaboliques
MCU-PH	LANDELLE Caroline	Bactériologie – virologie ; Hygiène hospitalière
PU-PH	LANTUEJOUL Sylvie	Anatomie et cytologie pathologiques
MCU-PH	LARDY Bernard	Biochimie et biologie moléculaire
MCU - PH	LE GOUELLEC Audrey	Biochimie et biologie moléculaire
PU-PH	LECCIA Marie-Thérèse	Dermato-vénéréologie
MCF Ass.MG	LEDOUX Jean-Nicolas	Médecine Générale
PU-PH	LEROY Vincent	Gastroentérologie ; hépatologie ; addictologie
PU-PH	LETOUBLON Christian	Chirurgie viscérale et digestive
PU-PH	LEVY Patrick	Physiologie
PU-PH	LONG Jean-Alexandre	Urologie
MCU-PH	LUPO Julien	Bactériologie-virologie
PU-PH	MAGNE Jean-Luc	Chirurgie vasculaire ; Médecine vasculaire
MCU-PH	MAIGNAN Maxime	Médecine d'urgence
PU-PH	MAITRE Anne	Médecine et santé au travail
MCU-PH	MALLARET Marie-Reine	Hygiène hospitalière
PU-PH	MALLION Jean-Michel	Cardiologie
MCU-PH	MARLU Raphaël	Hématologie ; Transfusion
MCU-PH	MAUBON Danièle	Parasitologie et mycologie
PU-PH	MAURIN Max	Bactériologie-virologie
MCU-PH	MC LEER Anne	Histologie, embryologie et cytogénétique
MCU-PH	MONDET Julie	Histologie, embryologie et cytogénétique
PU-PH	MORAND Patrice	Bactériologie-virologie
PU-PH	MOREAU-GAUDRY Alexandre	Biostatistiques, informatique médicale et technologies de communication
PU-PH	MORO Elena	Neurologie
PU-PH	MORO-SIBILOT Denis	Pneumologie
MCU-PH	MORTAMET Guillaume	Pédiatrie
PU-PH	MOUSSEAU Mireille	Cancérologie
PU-PH	MOUTET François	Chirurgie plastique, reconstructrice et esthétique ; brûlologie
MCF Ass.MG	ODDOU Christel	Médecine Générale
MCU-PH	PACLET Marie-Hélène	Biochimie et biologie moléculaire
PU-PH	PAILHE Régis	Chirurgie orthopédique et traumatologie
PU-PH	PALOMBI Olivier	Anatomie
PU-PH	PARK Sophie	Hématologie ; Transfusion
PU-PH	PASSAGGIA Jean-Guy	Anatomie
PR Ass.MG	PAUMIER-DESBRIERES Françoise	Médecine Générale
PU-PH	PAYEN DE LA GARANDERIE Jean-François	Anesthésiologie-réanimation et médecine péri-opératoire
MCU-PH	PAYSANT François	Médecine légale et droit de la santé
MCU-PH	PELLETIER Laurent	Biologie cellulaire
PU-PH	PELLOUX Hervé	Parasitologie et mycologie

CORPS	NOM-PRENOM	Discipline universitaire
PU-PH	PEPIN Jean-Louis	Physiologie
PU-PH	PERENNOU Dominique	Médecine physique et de réadaptation
PU-PH	PERNOD Gilles	Médecine vasculaire
PU-PH	PIOLAT Christian	Chirurgie infantile
PU-PH	PISON Christophe	Pneumologie
PU-PH	PLANTAZ Dominique	Pédiatrie
PU-PH	POIGNARD Pascal	Bactériologie-virologie
PU-PH	POLACK Benoît	Hématologie
PU-PH	POLOSAN Mircea	Psychiatrie d'adultes
PU-PH	RAMBEAUD Jean-Jacques	Urologie
PU-PH	RAY Pierre	Biologie et médecine du développement et de la reproduction
MCU-PH	RENDU John	Biochimie et biologie moléculaire
MCU-PH	RIALLE Vincent	Biostatistiques, informatique médicale et technologies de communication
PU-PH	RIETHMULLER Didier	Gynécologie-obstétrique ; gynécologie médicale
PU-PH	RIGHINI Christian	Oto-rhino-laryngologie
PU-PH	ROMANET Jean Paul	Ophtalmologie
PU-PH	ROSTAING Lionel	Néphrologie
MCU-PH	ROUSTIT Matthieu	Pharmacologie fondamentale ; pharmacologie clinique ; addictologie
MCU-PH	ROUX-BUISSON Nathalie	Biochimie et biologie moléculaire
MCF Ass.MG	ROYER DE VERICOURT Guillaume	Médecine Générale
MCU-PH	RUBIO Amandine	Pédiatrie
PU-PH	SARAGAGLIA Dominique	Chirurgie orthopédique et traumatologie
MCU-PH	SATRE Véronique	Génétique
PU-PH	SAUDOU Frédéric	Biologie cellulaire
PU-PH	SCHMERBER Sébastien	Oto-rhino-laryngologie
PU-PH	SCHWEBEL Carole	Médecine intensive-réanimation
PU-PH	SCOLAN Virginie	Médecine légale et droit de la santé
MCU-PH	SEIGNEURIN Arnaud	Epidémiologie, économie de la santé et prévention
PU-PH	STAHL Jean-Paul	Maladies infectieuses ; Maladies tropicales
PU-PH	STANKE Françoise	Pharmacologie fondamentale
MCU-PH	STASIA Marie-José	Biochimie et biologie moléculaire
PU-PH	STURM Nathalie	Anatomie et cytologie pathologiques
PU-PH	TAMISIER Renaud	Physiologie
PU-PH	TERZI Nicolas	Médecine intensive-réanimation
MCU-PH	TOFFART Anne-Claire	Pneumologie
PU-PH	TONETTI Jérôme	Chirurgie orthopédique et traumatologie
PU-PH	TOUSSAINT Bertrand	Biochimie et biologie moléculaire
PU-PH	VANZETTO Gérald	Cardiologie
PU-PH	VUILLEZ Jean-Philippe	Biophysique et médecine nucléaire
PU-PH	WEIL Georges	Epidémiologie, économie de la santé et prévention
PU-PH	ZAOUI Philippe	Néphrologie
PU-PH	ZARSKI Jean-Pierre	Gastroentérologie ; hépatologie ; addictologie

PU-PH : Professeur des Universités - Praticiens Hospitaliers
MCU-PH : Maître de Conférences des Universités - Praticiens Hospitaliers
PU-MG : Professeur des Universités de Médecine Générale
MCU-MG : Maître de Conférences des Universités de Médecine Générale
PR Ass.MG : Professeur des Universités Associé de Médecine Générale
MCF Ass.MG : Maître de Conférences Associé de Médecine Générale

8. SERMENT D'HIPPOCRATE



SERMENT D'HIPPOCRATE

En présence des Maîtres de cette Faculté, de mes chers condisciples et devant l'effigie d'HIPPOCRATE,

Je promets et je jure d'être fidèle aux lois de l'honneur et de la probité dans l'exercice de la Médecine.

Je donnerai mes soins gratuitement à l'indigent et n'exigerai jamais un salaire au dessus de mon travail. Je ne participerai à aucun partage clandestin d'honoraires.

Admis dans l'intimité des maisons, mes yeux n'y verront pas ce qui s'y passe ; ma langue taira les secrets qui me seront confiés et mon état ne servira pas à corrompre les mœurs, ni à favoriser le crime.

Je ne permettrai pas que des considérations de religion, de nation, de race, de parti ou de classe sociale viennent s'interposer entre mon devoir et mon patient.

Je garderai le respect absolu de la vie humaine.

Même sous la menace, je n'admettrai pas de faire usage de mes connaissances médicales contre les lois de l'humanité.

Respectueux et reconnaissant envers mes Maîtres, je rendrai à leurs enfants l'instruction que j'ai reçue de leurs pères.

Que les hommes m'accordent leur estime si je suis fidèle à mes promesses.

Que je sois couvert d'opprobre et méprisé de mes confrères si j'y manque.