厚生労働科学研究費補助金

第3次対がん総合戦略研究事業

消化器がん個別化医療におけるファンクショナルゲノミクス、プロテオミクス、メタボロミクスの臨床応用と治療体制の確立

平成21年度　総括・分担研究報告書

主任研究者　門田　守人

平成22（2010）年4月

目次

厚生労働科学研究費補助金（第３次対がん総合戦略研究事業）

総括研究報告書

消化器がん個別化医療におけるファンクショナルゲノミクス、プロテオミクス、メタボロミクスの臨床応用と治療体制の確立

研究代表者　　門田守人　　大阪大学・副学長

研究要旨

我々は基盤研究（A）の補助により、個別化医療の確立に向け、消化器がんの網羅的遺伝子発現解析手法を用いたファンクショナルゲノミクスによる生物学的特性の解明を進めてきた。今後は、遺伝子のみならず、タンパク、代謝産物を対象としたプロテオミクス、メタボロミクス技術も応用し、これまでの基礎研究成果を臨床応用するため、大規模症例を対象とした prospective な検証による evidence を示す必要がある。そこで、本３次がんの事業では、個別化医療実用化のための体制整備と臨床応用を目的として、臨床チップやバイオマーカーによる予後予測臨床試験を計画した。

A. 研究目的

2007 年度に実施されるがん対策基本法では、患者本人の意向を尊重した適切な医療体制の整備を基本理念とし、革新的技術を応用したがん研究の推進と成果の臨床応用が重要な課題として挙げられている。医療資源と患者の利益という観点より、evidence に基づいた治療の標準化は個別化とのダイナミックな循環によって進められるべきである。個別化医療に必要なのはまず個性の診断で、分子生物学的な特徴をあらゆるサンプルソースを用いて体系的に捉えることが有効である。そこで、本３次がんの事業では、消化器がんの大規模症例を対象に、がん組織の遺伝子・タンパク発現プロファイルによる転移・再発の予測診断系の構築と、がん患者の末梢血からメタボローム解析によるバイオマーカーを探索し、prospective な検証によって分子個別診断の evidence を示すとともに、その成果を臨床応用化することを目的とする。

B. 研究方法

MALDI-TOF/MS と安定同位体標識試薬 NBS (2-nitrobenzenesulfenyl)法を組み合わせたプロテオーム解析によって同定された各臓器別の特異的タンパクをウェスタンブロット法、IHC でそれぞれ発現を検証する。

さらに、より簡便性を図るため、がん患者の末梢血から超高感度・超高分解能のフーリエ変換質量分析とハイスループット・スクリーニング法(HTS)を用いてメタボライトを同時一斉分析し、メタボライトバイオマーカーを探索する。

（倫理面への配慮）

本研究ではゲノムは扱わないが、３省合同の「ゲノムに関する指針」に準じた情報管理を行い、大阪大学の倫理規定に従って、患者の同意が得られたサンプルを使用した。

C. 研究結果

大腸がん 24 例、肝臓がん 12 例を NBS 解析し、大腸がんで特異的に発現する新規 22 種類のタンパクおよび肝転移に関与する 12 種類のタンパク、また肝臓がんで特異的に発現する 64 種類のタンパクを同定した。さらに大腸がんの病勢を反映する 3 種の血清中タンパクをし、独立した 250 例の血清サンプルでその診断能を検証した。同定遺伝子発現は RT-PCR 法で、タンパク発現はウェスタンブロット法でそれぞれデータの信頼性を実証した。

メタボロミクスでは大腸がん 83 例、胃がん 45 例、膵がん 40 例の血清分析で、それぞれ 6 種、10 種、8 種の特異的なメタボライトを同定し、大腸がんの 200 例の検証試験では、血清存在診断で特異度 97%、感度 74%の高い正診率が得られた。

D. 考察

それぞれの OMIC 技術によって同定された

分子は、他の assay 法での発現 verification でも相同性の高いデータが得られていることから、それぞれがんの存在・病勢診断マーカー、さらには治療標的となることが期待される。今後は遺伝子とタンパクの相互関係を考慮し、パスウェイネットワーク解析することで、より中心的な役割を果たす分子の絞り込みと、candidate を適正に搭載した臨床型の DNA チップを用いた正確な予測診断系が期待される。また血清レベルでもメタボライトマーカーによる早期大腸がんリスクを評価する可能性が示され、OMICS 技術の臨床応用が期待された。

E. 結論

本研究では、がんの遺伝子・タンパクの両者から得られた基礎的研究の成果と、がん患者の末梢血のメタボローム解析によって得られた特異的メタボライト発現パターンの結果を、臨床研究デザインに合わせ prospective に解析することで、トランスレーショナルリサーチとして十分な evidence が得られることが期待され、臨床応用化の基盤が整えられてきた。

F. 研究発表

1. 論文発表

1)Yoshioka S., Takemasa I., Nagano H., Kittaka N., Noda T., Wada H., Kobayashi S., Marubashi S., Yakeda Y., Umeshita K., Dono K., Matsubara K., Monden M.: Molecular predicition of early recurrence after resection of hepatocellular carcinoma. Br J Cancer 145(5):457-464 2009

2)Noda T., Nagano H., Takemasa I., Yoshioka S., Murakami M., Wada H., Kobayashi S., Marubashi S., Takeda Y., Dono K., Umeshita K., Matsuura N., Matsubara K., Doki Y., Mori M., Monden M.: Activation of Wnt/beta-catenin signalling pathway induces chemoresistance to interferon-alpha/5-fluorouracil combination therapy for hepatocellular carcinoma.:Br J Cancer 100(10) : 1647-1658 2009

3)

4)Makino T., Yamasaki M., Takemasa I., Takeno A., Nakamura Y., Miyata H., Takiguchi S., Fujiwara Y., Matsuura N., Mori M., Doki Y.: Dickkopf-1 expression as a marker for predicting clinical outcome in esophageal squamous cell carcinoma.: Ann Surg Oncol. 16(7) : 2058-2064 2009

5)Motoori M. Takemasa I., Yamasaki M., Komori T., Takeno A., Miyata H., Takiguchi S., Fujiwara Y., Yasuda T., Yano M., Matsuura N., Matsubara K., Monden M., Mori M., Doki Y. : The feasibility of using biopsy samples from esophageal cancer for comprehensive gene expression profiling.:Int J Oncol 35(2) : 265-271 2009

6)Uemura M., Ikeda M., Sekimoto M., Haraguchi N., Mizushima T., Yamamoto H., Takemasa I., Ishii H,. Mori M.: Prevention of severe pelvic abscess formation following extended radical surgery for locally recurrent rectal cance .:Ann Surg Oncol 16:2204-2210 2009

7)Asaoka T., Kato T., Marubashi S., Dono K., Hama N., Takahashi H., Kobayashi S., Takeda Y., Takemasa I., Nagano H., Yoshida H., Ruiz P., Tzakis AG., Matsubara K., Monden M., Doki Y., Mori M.: Differential transcriptome patterns for acute cellular rejection in recipients with recurrent hepatitis C after liver transplantation.:Liver Transpl 15(12) : 1738-1749 2009

8)竹政伊知朗、石井秀始、原口直紹、三森功士、田中文明、永野浩昭、関本貢嗣、土岐祐一郎、森正樹.: 消化器癌における癌幹細胞研究の現状.: 日本外科学会雑誌

110(4):207-212 2009

9)植村守、山本浩文、原口直紹、竹政伊知朗、水島恒和、池田正孝、石井秀始、関本貢嗣、土岐祐一郎、森正樹.: 大腸癌に対する化学療法、分子標的治療.: 総合臨床 58(9) : 1965-1971 2009

2. 学会発表

1)Asaoka T., Marubashi S., Dono K., Takahashi T., Kobayashi S., Takeda Y., Takemasa I., Nagano H., Ruiz P., Doki Y., Mori M.: Evaluation of potential allograft rejection biomarkers after liver transplantion.: American Journal of Transplantation.2009 年 5 月(Boston)

2)金浩敏、竹政伊知朗、原口直紹、水島恒和、池田正孝、石井秀始、山本浩文、関本貢嗣、土岐祐一郎、森正樹.: 腹腔鏡下手術における#253 リンパ節郭清についての検討.: 日本外科学会定期学術集.2009 年 4 月(福岡)

3)山本浩文、小西健、鈴木玲、原口直紹、水島恒和、竹政伊知朗、池田正孝、石井秀始、関本貢嗣、土岐祐一郎、森正樹.: 癌と間質の interface における分子解析と治療応用.: 癌と間質の interface における分子解析と治療応用. 日本外科学会定期学術集.2009 年 4 月(福岡)

4)野田剛広、永野浩昭、竹政伊知朗、富丸慶人、村上昌裕、小林省吾、丸橋繁、武田裕、種村匡弘、北川透、堂野恵三、梅下浩司、松原謙一、門田守人、土岐祐一郎、森正樹.: IFN 併用化学療法の感受性における Wnt/beta-catenin シグナルの関与と感受性マーカーの同定.: 日本外科学会定期学術集.2009 年 4 月(福岡)

5)徳岡優佳、竹政伊知朗、渡辺真、三宅正和、水島恒和、池田正孝、山本浩文、関本貢嗣、西村紀、門田守人、森正樹.: NBS 法による大腸癌組織を用いた新規肝転移関連蛋白の検討.: 日本外科学会定期学術集.2009 年 4 月(福岡)

6)永野浩昭、丸橋繁、竹政伊知朗、小林省吾、武田裕、種村匡弘、北川透、堂野恵三、土岐祐一郎、森正樹、門田守人.: 肝癌外科治療後の再発予測・予防における臨床・基礎両面からの個別化治療への可能性.: 日本肝臓学会総会.2009 年 6 月 (神戸)

7)野田剛広、永野浩昭、竹政伊知朗、富丸慶人、村上昌裕、小林省吾、丸橋繁、武田裕、種村匡弘、北川透、堂野恵三、梅下浩司、松浦成昭、松原謙一、門田守人、土岐祐一郎、森正樹.: 肝細胞癌に対する IFN 併用化学療法の抵抗性における Wnt/beta-catenin シグナルの関与.:日本肝臓学会総会.2009 年 6 月 (神戸)

8)牧野知紀、山崎誠、竹政伊知朗、宮田博志、瀧口修司、藤原義之、中島清一、西田俊朗、森正樹、土岐祐一郎.: 食道扁平上皮癌におけるサイトケラチン 18 (CK18) 発現の定義.: 日本外科学会定期学術集.2009 年 4 月(福岡)

9)植村守、山本浩文、原口直紹、竹政伊知朗、水島恒和、池田正孝、石井秀始、関本貢嗣、土岐祐一郎、森正樹.: 生体内低酸素誘導遺伝子検索モデルとしての大腸癌肝転移巣の有用性.:日本消化器癌発生学会総.2009 年 11 月(広島)

G. 知的財産権の出願・登録状況

(予定を含む。)

1.特許取得

特になし

2.実用新案登録

特になし

3.その他

特になし

厚生労働科学研究費補助金（第３次対がん総合戦略研究事業）

分担研究報告書

消化器がん個別化医療におけるファンクショナルゲノミクス、プロテオミクス、メタボロミクスの臨床応用と治療体制の確立

分担研究者　松原謙一　株式会社ＤＮＡチップ研究所

研究要旨

消化器がん個別化医療におけるファンクショナルゲノミクスの確立

A. 研究目的

大腸がんを対象に用いてファンクショナルゲノミクスを基に実用化に向けた研究開発を行う。

B. 研究方法

切除大腸がん試料からＲＮＡを抽出し発現プロファイルを解析することにより術後の個別化予後予測を可能とする。これまでの成果の上に、さらに多数の試料を解析して成果確認を行う一方、より実用に適する方法論を開発する。

（倫理面への配慮）

大阪大学生命研究倫理委員会の承認済

C.　研究結果

昨年度に行った大腸がん stageII 手術試料にさらに 160 試料を追加し、予後予測が適正に行われることを確認した。さらにチップ基板を安価なものに取り換えても成果の劣らないことを確認した。

D. 考察

本解析を進めることにより、がん切除患者の予後予測が行えることが証明された。内容・技術共に実用に適するレベルに達したので、今後大規模実地テストに進むことができる。

E. 結論

大腸がん切除試料のＲＮＡを多数解析しそのプロファイルの特徴から、少なくとも StageⅡの患者に対して実用レベルで予後予測に進むことができることを確認した。また、解析技法の検討を行い実用に適する新たなチップ基板の検討を行い順調に推移した。

F. 研究発表

1. 論文発表

1)M Miyake., I Takemasa., R Matoba., M Tanino., S Niijima., M Ikeda., H Yamamoto., M Sekimoto, S Kuhara., T Okayama., M Monden., K Matsubara ., M Mori.,Heterogeneity of Colorectal Cancers and Extraction of Discriminator Gene Signatures for Personalized Prediction of Prognosis. Brit. J. Cancer 投稿中

2. 学会発表

なし

G. 知的財産権の出願・登録状況

（予定を含む。）

1.特許取得

(準備中)

2.実用新案登録

特になし

3.その他

特になし

厚生労働科学研究費補助金（第３次対がん総合戦略研究事業）

分担研究報告書

NBS法による大腸癌組織の蛋白質発現解析

研究分担者　　西村紀　大阪大学蛋白質研究所

研究要旨

大腸癌組織のプロテオーム解析により同定された新規の大腸癌関連蛋白質のうち、ELISA 測定系が構築できた 20 種類について、大腸癌患者および健常者血漿中の蛋白質濃度を測定し、血中マーカーとしての利用可能性を評価した。その結果、３種類の蛋白質(Protein X, Protein Y, Protein Z)について、癌患者群と健常者群の間で有意な差が認められた。そこで、これら３つのマーカーについて、血中濃度と癌の進行度との関連性、術前・術後における血中濃度変化、マーカー閾値の設定と病態別陽性率などについて、さらに詳細な解析を行った。

これらの詳細解析の結果をまとめると、Protein X と Protein Y については存在診断マーカー、Protein Z については病勢マーカーとしての特徴をそれぞれ有しており、これら３種類の大腸癌関連蛋白質はいずれも臨床マーカーとして応用できる可能性があることがわかった。特に Protein Z については、既存の病勢マーカーである CEA, CA19-9 と

A. 研究目的

新規大腸癌関連蛋白質を同定し、バイオマーカー(診断、予後予測、治療ターゲットなど)への応用を目的とする。

B. 研究方法

ELISA (Enzyme-Linked ImmunoSorbent Assay)による血中濃度測定によって癌患者群(105 症例)と健常者群(100 名)間で統計学的有意差($p<0.05$)が認められた３種類(Protein X, Protein Y, Protein Z)の癌関連蛋白質について、①癌の進行度(Tumor Stage)と血中濃度の関係性　②術前・術後における血中濃度変化　③癌の病態別でのマーカー陽性率、について詳細解析を行った。

Protein Z については病勢マーカーとしての特徴を示していたことから、CEA および CA19-9 に対する相補的マーカーとしての有用性を検討した。

なお、本研究では既存の癌マーカー値(ＣＥＡ、ＣＡ１９－９、ＳＣＣ抗原、ＣＡ１２５、ＣＡ１５－３及びＰＳＡ)が全て正常範囲である者を健常人として定義して各解析を行った。また、術前・術後での血中濃度の比較においては、手術後の根治度が A (Cure A)であった患者の血漿サンプルを解析対象とした。

（倫理面への配慮）

大阪大学医学部の倫理規定に従って患者の同意が得られたサンプルを使用した。

C. 研究結果

各検体における血中濃度と癌の進行度との関係について調べると、Protein Z の血中濃度は Tumor stage の進行に伴って増加する傾向を示していたのに対して、Protein X と Protein Y についてはそのような傾向を示していなかった。また、術前・術後における血中濃度比較では、3 種類全ての癌関連蛋白質について、術後に濃度が有意($p<0.05$)に減少していることが確認された。

次に、各癌関連蛋白質のマーカーとしての閾値設定を、ROC 曲線を利用して行った。この値を用いて癌の病態別(locoregional stage (stage 0-II)、metastatic stage (stage III-IV))でのマーカー陽性率について調べたところ、Protein X と ProteinY については比較的早期ステージである Locoregional stage 患者群での陽性率が高いのに対して、Protein Z については metastatic stage 患者群での陽性率が高かった。

特に ProteinZ については、既存の大腸癌マーカーである CEA, CA19-9 と併用することにより、CEA, CA19-9 を単独で使用した場合と比較して陽性率の十分な上積み効果が認められた。

D. 考察

Protein Z については、血中濃度は CEA と同様に Tumor stage の進行に伴って増加する傾向を示していた。また、術前術後の比較においても Cure A 患者のほとんどにおいて、その値が減少していた。これらの性質は、CEA のような病勢マーカーに特徴的なもの

であり、Protein Zもまた病勢マーカーであることを強く示唆する結果である。
また、Protein Zの既存マーカー(CEAおよびCA19-9)に対する相補的マーカーとしての有用性を検討すると、Protein Zを組み合わせた場合の上積み効果が十分あることが分かった。つまり、Protein ZをCEAやCA19-9と組み合わせて用いることにより、術後の経過観察を、より多くの患者に対して行える可能性が示された。
その一方、Protein XとProtein Yについては、Tumor stageが進行するに伴って濃度が増加する傾向を示していなかった。病勢別での陽性率について調べてみると、Metastatic stageの患者の陽性率は低く、むしろLocoregional stageの患者での陽性率の方が高いほどであった。以上のことから、Protein XとProtein Yは存在診断に有利な特徴を備えていると思われる。

E. 結論

我々は今回、20種類の大腸癌マーカー候補蛋白質のELISA解析により、3種類の癌関連蛋白質の血漿濃度が大腸癌の罹患と関連性があることを初めて実証した。更に、Protein Zに関しては病勢マーカーとしての特徴、Protein X, Protein Yは存在診断マーカーとしての特徴を呈していることが分かった。特に、Protein Zに関してはCEA、CA19-9に対する相補的病勢マーカーとしての応用の可能性も示唆された。

F. 研究発表

1)Sonomura K,. Nishimura O et al.: Terminal proteomics: N- and C-terminal analysis for high-fidelity identification of proteins using mass spectrometry.: Rapid Communications in Mass Spectrometry (23):611-618,2009

2)Matsuo E, Nishimura O et al.: A new strategy for protein biomarker discovery utilizing 2-nitrobenzenesulfenyl (NBS) reagent and its applications to clinical samples.: Journal of chromatography B(877):2607-2614,2009

3)Matsuo E, Nishimura O et al., A new strategy for protein biomarker discovery utilizing 2-nitrobenzenesulfenyl (NBS) reagent and its applications to clinical samples.: Proteomics(9):4063-4070,2009

4)Sonomura K, Nishimura O et al., A method for terminus proteomics: Selective isolation and labeling of N-terminal peptide from protein through transamination reaction.: Bioorganic & Medicinal Chemistry Letters(19):6544-6547,2009

5)Ezure T, Nishimura O et al., Expression of human Cu, Zn-superoxide dismutase in an insect cell-free system and its structural analysis by MALDI-TOF MS.: Journal of biotechnology(144):287-292,2009

6)Suzuki T, Nishimura O et al.,Preparation of ubiquitin-conjugated proteins using an insect cell-free protein synthesis system.: Journal of biotechnology(145):73-78,2009

7)Omar MFM, Nishimura O et al., Molecular-assisted immunohistochemical optimization.: Acta histochemica: in press,2010

8)Kuyama H, Nishimura O et al.: An approach to C- and N-terminal sequencing of protein: Mass spectrometry-based de novo amino acid sequencing with the combination of site-specific tris (2,4,6-trimethoxyphenyl)phosphonium-acetylation of a-amino group and selective recovery of terminal peptides.: Biomacromolecular Mass Spectrometry(2) in press,2010

9)Ezure T, Nishimura O et al., Development of an Insect Cell-Free System.: Current Pharmaceutical Biotechnology in press,2010

G. 知的財産権の出願・登録状況

(予定を含む。)

1.特許取得

現在、特許申請準備中(２件)

2.実用新案登録

特になし

3.その他

特になし

厚生労働科学研究費補助金（第３次対がん総合戦略研究事業）

分担研究報告書

消化器がんと関連するバイオマーカーの探索・評価

研究分担者　　山崎　泰代　Phenomenome Discoveries Inc.(PDI)

研究要旨

消化器がん患者の末梢血からメタボローム解析によりバイオマーカーを探索し、臨床応用を目的として大規模症例を対象とした評価試験を行った。

A. 研究目的

消化器がん患者の末梢血からメタボローム解析によりバイオマーカーを探索し、臨床応用化することを目的とする。

B. 研究方法

がん患者の末梢血から、PDI 独自のフーリエ変換イオンサイクロトロン共鳴質量分析計を用いたメタボロミクス技術を用いて、独自のデータ解析を行い、メタボライトバイオマーカーを探索した（フェーズ 1）。

さらに上記探索研究から発見された血中バイオマーカー候補を測定する、多検体分析が可能なスクリーニング法を開発し、盲検体を用いて、バイオマーカーとしての臨床評価を行った（フェーズ 2）。

フェーズ 1 では、40 症例（ステージ I～IVa, IVb）の膵がん患者、50 例の正常対照群の末梢血検体が、メタボローム解析のために提供された。

フェーズ 2 では、351 例（含；膵がん、健常対照群）の末梢血が、バイオマーカー候補の分析のため、ブラインドで提供された。

（倫理面への配慮）

大阪大学の倫理規定に従って被験者の同意が得られた検体を使用した。

C. 研究結果

計 90 例の末梢血のメタボローム解析により、膵がん関連メタボライト群を発見した。検出された膵がん関連メタボライト群のうち、水酸化超長鎖多価不飽和脂肪酸群、各種リン脂質群に関して、多検体分析が可能なスクリーニング法を開発した。

続いて計 90 例の末梢血（フェーズ 1）を、上記スクリーニング法を用いて分析し、境界値を求めた。計 351 例の末梢血（フェーズ 2）をブラインドの状態で分析し、上記境界値を用いて検証したところ、バイオマーカー候補 1 では、特異度 95%、感度 70%が得られ、候補 2 では、特異度 98%、感度 56%の正診率が得られた。

D. 考察

膵がんは早期発見が難しく、進行してからでないと見つからないため治りにくいといわれている。今回の発見・検証により、膵がんのリスク判定バイオマーカーとして臨床応用の可能性が示され、早期発見に繋がるものと期待される。

今後、各種候補メタボライトのスクリーニング法を用いた分析を行い、その組合せにより、さらに有用なリスク判定ツールとなる可能性がある。

E. 結論

膵がん特異的に発現しているメタボライトバイオマーカー群は、その組合せにより新規のスクリーニング法としての応用が今後期待される。

F. 研究発表

1. 論文発表

1) Ritchie S.A., Ahiahonu W.K.A., Jayasinghe D., Heath D., Liu J., Lu Y., Jin W., Kavianpour A., Yamazaki Y., Khan A.M., Hossain M., Su-Myat K.K., Wood L.P., Krenitsky K., Takemasa I., Miyake M., Sekimoto M., Monden M., Matsubara H., Nomura F., Goodenowe B.D.; Reduced levels of hydroxylated, polyunsaturated ultra long-chain fatty acids in the serum of colorectal cancer patients: implications for early screening and detection.: BMC Medicine 8(13) 2010.

2. 学会発表

1) Yamazaki Y., Pastural É., Ritchie S., Goodenowe D., Akita H., Takeda Y., Takemasa I., Nagano H., Mori M., Doki Y., Monden M.: Identification of a metabolite profile specific to pancreatic cancer.: 第 68 回日本癌学会学術

総会，2009.10.1-. 10.3　（横浜）

G. 知的財産権の出願・登録状況
（予定を含む。）
1.特許取得
　特になし
2.実用新案登録
　特になし
3.その他
　特になし

研究成果の刊行に関する一覧表

書籍　(外国語)

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
Kuyama H, Nishimura O *et al.*	A New Method for N- and C-Terminal De Novo Amino Acid Sequencing of Proteins By Mass Spectrometry	J.K.Lang	Handbook on Mass spectrometry: Instrumentation, Data and Analysis, and Applications	Nova Science Publishers, Inc.	New York, USA	2010	389-420
Sonomura K, Nishimura O *et al.*	SPECIFIC ISOLATION OF N- AND C-TERMINAL PEPTIDES OF PROTEINS THOROUGH A TRANSAMINATION REACTION	Kouji Okamoto	PEPTIDE SCIENCE 2009	Japanese Peptide Symposium Kitakyushu	Osaka, Japan	2010	in press

雑誌　(外国語)

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Yoshioka S, et.al	Molecular predicition of early recurrence after resection of hepatocellular carcinoma	Br J Cancer	145(5)	457-464	2009
Noda T, et.al	Activation of Wnt/beta-catenin signalling pathway induces chemoresistance to interferon-alpha/5-fluorouracil combination therapy for hepatocellular carcinoma.	Br J Cancer	100(10)	1647-1658	2009
Makino T, et.al	Dickkopf-1 expression as a marker for predicting clinical outcome in esophageal squamous cell carcinoma	Ann Surg Oncol	16(7)	2058-2064	2009
Motoori M, et.al	The feasibility of using biopsy samples from esophageal cancer for comprehensive gene expression profiling	Int J Oncol	35(2)	265-271	2009
Uemura M, et.al	Prevention of severe pelvic abscess formation following extended radical surgery for locally recurrent rectal cance	Ann Surg Oncol	16	2204-2210	2009
Asaoka T, et.al	Differential transcriptome patterns for acute cellular rejection in recipients with recurrent hepatitis C after liver transplantation	Liver Transpl	15(12)	1738-1749	2009

研究成果の刊行に関する一覧表

M Miyake, K Matsubara *et al.*	Heterogeneity of Colorectal Cancers and Extraction of Discriminator Gene Signatures for Personalized Prediction of Prognosis	Brit. J. Cancer		（投稿中）	
Matsuo E, Nishimura O *et al.*	A new strategy for protein biomarker discovery utilizing 2-nitrobenzenesulfenyl (NBS) reagent and its applications to clinical samples	Journal of chromatography B	877	2607-2614	2009
Kuyama H, Nishimura O *et al.*	An approach for detecting C-terminal amidation of proteins and peptides by mass spectrometry in conjunction with chemical derivatization	Proteomics	9	4063-4070	2009
Sonomura K, Nishimura O *et al.*	A method for terminus proteomics: Selective isolation and labeling of N-terminal peptide from protein through transamination reaction	Bioorganic & Medicinal Chemistry Letters	19	6544-6547	2009
Ezure T, Nishimura O *et al.*	Expression of human Cu, Zn-superoxide dismutase in an insect cell-free system and its structural analysis by MALDI-TOF MS.	Journal of biotechnology	144	287-292	2009
Suzuki T, Nishimura O *et al.*	Preparation of ubiquitin-conjugated proteins using an insect cell-free protein synthesis system.	Journal of biotechnology	145	73-78	2010
Omar MFM, Nishimura O *et al.*	Molecular-assisted immunohistochemical optimization	Acta histochemica		in press	2010
Kuyama H, Nishimura O *et al.*	An approach to C- and N-terminal sequencing of protein: Mass spectrometry-based *de novo* amino acid sequencing with the combination of site-specific tris (2,4,6-trimethoxyphenyl) phosphonium-acetylation of α-amino group and selective recovery of terminal peptides	Biomacromolecular Mass Spectrometry		in press	2010
Ezure T, Nishimura O *et al.*	Development of an Insect Cell-Free System.	Current Pharmaceutical Biotechnology		in press	2010
Ritchie SA, et.al	Reduced levels of hydroxylated, polyunsaturated ultra long-chain fatty acids in the serum of colorectal cancer patients: implications for early screening and detection	BMC Medicine	8	13	2010

研究成果の刊行に関する一覧表

書籍（日本語）

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

雑誌（日本語）

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
竹政伊知朗，他	消化器癌における癌幹細胞研究の現状	日本外科学会雑誌	110(4)	207-212	2009
植村守，他	大腸癌に対する化学療法、分子標的治療	総合臨床	58(9)	1965-1971	2009
松尾英一，西村紀，他	NBS解析システムの開発からバイオマーカ候補タンパク質の探索へ	島津評論	66	161-172	2010
中島ちひろ，西村紀，他	質量分析によるタンパク質・ペプチドC末端アミド化解析のための化学的誘導体化を用いた新規手法について	島津評論	66	185-194	2010

EUROPEAN JOURNAL OF CANCER 45 (2009) 881–889

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejconline.com

Molecular prediction of early recurrence after resection of hepatocellular carcinoma

Shinichi Yoshioka[a], *Ichiro Takemasa*[a,*], *Hiroaki Nagano*[a], *Nobuyoshi Kittaka*[a], *Takehiro Noda*[a], *Hiroshi Wada*[a], *Shogo Kobayashi*[a], *Shigeru Marubashi*[a], *Yutaka Takeda*[a], *Koji Umeshita*[a], *Keizo Dono*[a], *Kenichi Matsubara*[b], *Morito Monden*[a]

[a]*Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka E-2, Suita, 565-0871 Osaka, Japan*
[b]*DNA Chip Industry Co., Ltd., Tokyo, Japan*

ARTICLE INFO

Article history:
Received 16 October 2008
Received in revised form
11 December 2008
Accepted 15 December 2008
Available online 23 January 2009

Keywords:
Hepatocellular carcinoma
Gene expression profile
Intrahepatic recurrence

ABSTRACT

The prognosis of hepatocellular carcinoma (HCC) remains poor. Vascular invasion, tumour multiplicity and large tumour size are the conventional poor prognostic indicators related to early recurrence. However, it is difficult to predict prognosis of each HCC in the absence of these indicators. The purpose of this study is to predict early recurrence of HCC after radical resection based on whole human gene expression profiling. Microarray analyses were performed in 139 HCC primary tumours. A total of 88 cases lacking the conventional poor prognostic indicators were analysed to establish a molecular prediction system characteristic for early recurrence in 42 training cases with two polarised prognoses, and to test its predictive performance in 46 independent cases (group C). Subsequently, this system was applied to another 51 independent cases with some poor prognostic indicators (group D). The molecular prediction system accurately differentiated HCC cases into poor and good prognoses in both the independent group C (disease-free survival [DFS]: $p = 0.029$, overall survival [OS]: $p = 0.0043$) and independent group D (DFS: $p = 0.0011$, OS, $p = 0.035$). Multivariate Cox regression analysis indicated that the clinical value of molecular prediction system was an independent prognostic factor ($p < 0.0001$, hazard ratio = 3.29). Gene expression pattern related to early intrahepatic recurrence inherited in the primary HCC tumour can be useful for the prediction of prognosis.

1. Introduction

Hepatocellular carcinoma (HCC) is a common malignancy worldwide and is currently the third major cause of cancer-related deaths in Japan.[1] Recent progress in diagnostic and treatment technologies has improved the long-term survival of patients with HCC, but the prognosis remains unfavourable. Surgical resection has been one of the mainstays in curative treatment of HCC. However, even after curative resection, 80% of patients develop intrahepatic recurrence and 50% die within 5 years.[2,3]

Some patients who have undergone curative resection suffer an unpredictable early fulminant recurrence in the remnant liver, and this is associated with dismal prognosis. Detection of cases with early recurrence at the time of resection is beneficial for better decision making for treatment. In this regard, a staging system for HCC according to clinicopathological findings has been applied to assess the risk of recurrence following resection.[4]

Vascular invasion, tumour multiplicity and large tumour size (tumours measuring more than 5 cm in diameter) are poor prognostic indicators of HCC,[2,5–7] and it is difficult to

* *Corresponding author:* Tel.: +81 6 6879 3251; fax: +81 6 6879 3259.
E-mail address: alfa-t@sf6.so-net.ne.jp (I. Takemasa).
0959-8049/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ejca.2008.12.019

predict the prognosis of each case of HCC in the absence of these conventional indicators. However, the above-mentioned poor prognostic indicators are insufficient to predict the recurrence of HCC patients who undergo curative resection[21], thus new indicators are sought to help predict early intrahepatic recurrence developing after surgery in these patients.

Carcinogenesis is regulated by various changes on a genetic level, and several studies have discussed the phenomenon of cancer metastasis based on the analysis of various molecules. While it is useful to understand cancer progression, it is difficult to predict early recurrence with the analysis of a single molecule. The reason is that recurrence might be regulated by multiple molecular changes and interactions, and it might be difficult to explain the phenomenon of recurrence of HCC by a single molecule.[8–10] Therefore, it is important to conduct a comprehensive analysis of these molecules. The approach of microarray technology provides considerable information on cancer features and behaviour in individuals in several malignant tumours.[11–14] Several molecular and genetic studies have been reported on the progression of HCC and prediction of response of chemotherapy,[15–18] and some concluded that the specific gene expression patterns in HCC cancerous tissues could predict early intrahepatic recurrence.[19–22] However, it is still challenging to detect early recurrence tumours at the time of resection due to the complex pathogenesis of HCC. A recent study suggested that the strict selection of a homogeneous training set of patients in building the classifiers is essential to improve the predictability, reproducibility and validity of classifiers.[23]

In the present study, whole gene analysis was performed using a more clearly and strictly defined design set taking account of the complex pathogenic process of HCC, which reflected the prognosis more directly than previous reports with larger number of analyses.[24]

2. Materials and methods

2.1. Patients

A total of 139 HCC patients who had undergone hepatectomy at the Osaka University Hospital were enrolled in this study. All patients were followed up after resection for at least 3 months and the median follow-up time of survival cases in this study was 36 months (range, 12–87 months). Informed consent was obtained from all patients to use their surgical specimens and the clinicopathological data for research purposes. Histological classification was based on the Edmondson grading system and clinical stage was determined according to the Cancer of the Liver Italian Programme (CLIP). A mixture of RNA from the normal parts of liver specimens of seven patients with liver metastases from intestinal carcinomas was used as a reference for microarray analysis. None of the reference cases had hepatitis B or C (HBV or HCV, respectively) infection and their liver function tests were within normal values. All tissues were snap-frozen into liquid nitrogen and were stored at −80 °C.

2.2. Experimental design

Fig. 1 illustrates schematically our experimental design. Prediction of early recurrence in patients lacking the above-mentioned conventional poor prognostic indicators is clinically beneficial. In our study, we analysed patients lacking the aforementioned poor prognostic indicators to solve such a problem. To select the informative genes that are related to the phenomenon of early recurrence, we used two groups with polarised time course during the training phase. One group (group A) comprised cases with poor prognosis ($n = 21$), representing patients who developed multiple

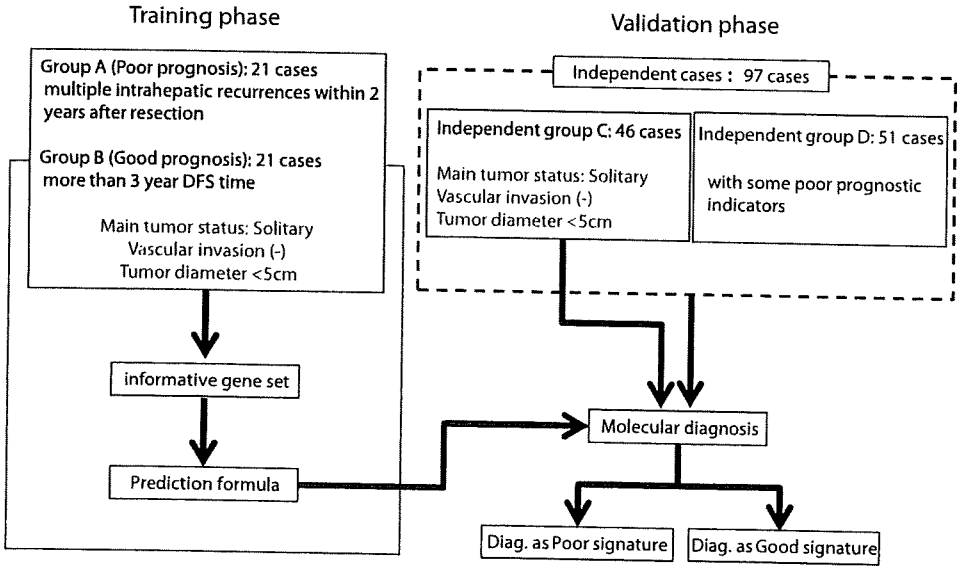

Fig. 1 – Schematic diagram of the experimental protocol. A molecular prediction system was constructed in the training phase. In the next step (validation phase), we applied this system to the independent group C ($n = 46$) and the entire group of independent cases ($n = 97$) comprising group C ($n = 46$) and group D ($n = 51$). Cases in grey coloured zones (Groups A, B and C) had similar clinicopathological conditions.

intrahepatic recurrences within 2 years after resection of the primary HCC. The second group (group B) comprised patients with satisfactory prognosis ($n = 21$), defined as more than 3-year disease-free survival (DFS) time. Table 1 summarises the clinicopathological features of patients of the two groups during the training phase. There were no differences between the two groups with regard to liver function tests and other clinicopathological variables except for the range of protein induced by vitamin K absence or antagonist II (PIVKA-II).

Based on the studies conducted in the training phase, a molecular prediction system was constructed using a set of informative genes. In the next step, we applied this system to another (independent) group C without any poor prognostic indicators as well ($n = 46$). The prediction system classified patients of group C into a 'poor signature' group (gene expression pattern resembled that of cases with poor prognosis) and a 'good signature' group (gene expression pattern resembled that of cases with good prognosis). Subsequently, we applied the prediction system to the independent group D ($n = 51$), which was composed of cases with positive status of some poor prognostic indicators. Finally, the independence of the diagnostic value of the molecular prediction results was verified by univariate and multivariate analyses using the whole independent cases, comprising patients of groups C and D.

2.3. *Microarray analysis*

Total RNA was extracted using TRIzol agent (Invitrogen, Carlsbad, CA), according to the instructions supplied by the manufacturer. Next, 2 μg of total RNA was used to synthesise double-strand cDNA that contained a promoter for T7 RNA polymerase. Amplified antisense RNA was synthesised by *in vitro* transcription of the cDNA templates by using the Amino Allyl MessageAmp aRNA kit (Ambion, Austin, TX). The reference and test sample were labelled with Cy3 and Cy5, mixed and hybridised on a microarray, AceGene Human oligo chip (DNA chip Research and Hitachi Software, Yokohama, Japan) DNA microarray. DNA microarray was used according to the instructions provided by the manufacturer (http://www.dna-chip.co.jp/thesis/AceGeneProtocol.pdf).

2.4. *Data analysis for postscanning*

The microarrays were scanned using ScanArray Lite and signal values were calculated using DNASIS array software (Hitachi Software Engineering Co., Yokohama, Japan). The local background was subtracted from each spot, and the ratio of the intensity of fluorescence from the Cy5 channel to the intensity of fluorescence from the Cy3 channel was calculated

Table 1 – Clinicopathological variables during the training phase.

	Poor prognosis group A ($n = 21$)	Good prognosis group B ($n = 21$)	P Value
Sex			
M	18	15	
F	3	6	0.452
Age, years			
<65	10	11	>0.999
⩾65	11	10	
HB infection			
+ve	8	11	0.535
–ve	13	10	
HC infection			
+ve	12	15	0.520
–ve	9	6	
Liver status			
Child A	19	15	0.239
Child B	2	6	
Tumour diameter, cm; mean (SD)	2.7 (0.8)	2.7 (1.1)	0.849•
AFP			
<400 ng/ml	18	18	>0.999
⩾400 ng/ml	3	3	
PIVKA-II			
<45 AU/ml	14	20	0.049
⩾45 AU/ml	7	1	
Capsule formation			
–ve	6	11	0.209
+ve	15	10	
Edmondson Grade			
I/II	13	16	0.504
III/IV	8	5	

P Values were calculated by the chi-square test, or by •Student *t*-test.

for each spot. Spots with intensity levels below the limit value were omitted. The ratio of expression level of each gene was converted to a logarithmic scale (base 2), and the data matrix was normalised to a median of 0 by standardising each sample.

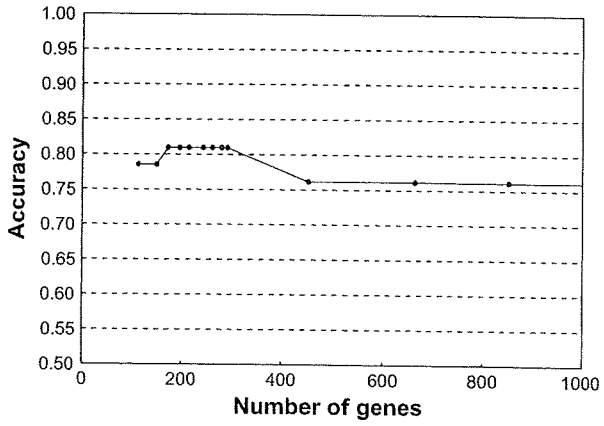

Fig. 2 – The accuracy curve based on weighted-voting algorithm with a leave-one-out cross validation. The accuracies in diagnosis of groups [ordinate] were plotted against the degree of *p*-value [abscissa]. The 172-gene set [P = 0.0004] marked the top accuracy. The accuracy was 80.2%. The *P* value was calculated by 10,000 times permutation test.

Genes with more than 15% missing data values in all samples in the training phase were excluded from the analysis. Missing data were compensated by averaging the expression data of 42 cases in the training phase.

2.5. *Construction of prediction system using gene expression patterns*

To detect the significant genes for prediction, we used permutation testing.[20] The original score of each gene (signal-to-noise ratio, $Si = (\mu A - \mu B)/(\sigma A + \sigma B)$, where μ and σ represent the mean and standard deviation of expression for each class, respectively) was calculated without permuting labels (responder or non-responder). The labels were randomly swapped and the values of S2N were calculated for the two groups. Repetition of this permutation 10,000 times provided a data matrix nearly the same as normal distribution. For each gene, the *P* value was calculated for the original S2N ratio with reference to the distribution of permuted data matrix. This model was evaluated by leave-one-out cross validation and the accuracy of each gene set was calculated based on the *P* value of the genes. As a supervised classification method, we adopted a weighted-voting (WV) algorithm.[13,14,19–22,25] We determined the optimal *P* value of the genes and classifier and constructed the prediction formula.

2.6. *Statistical analysis*

Clinicopathological indicators were compared using chi-square test and continuous variables were compared using

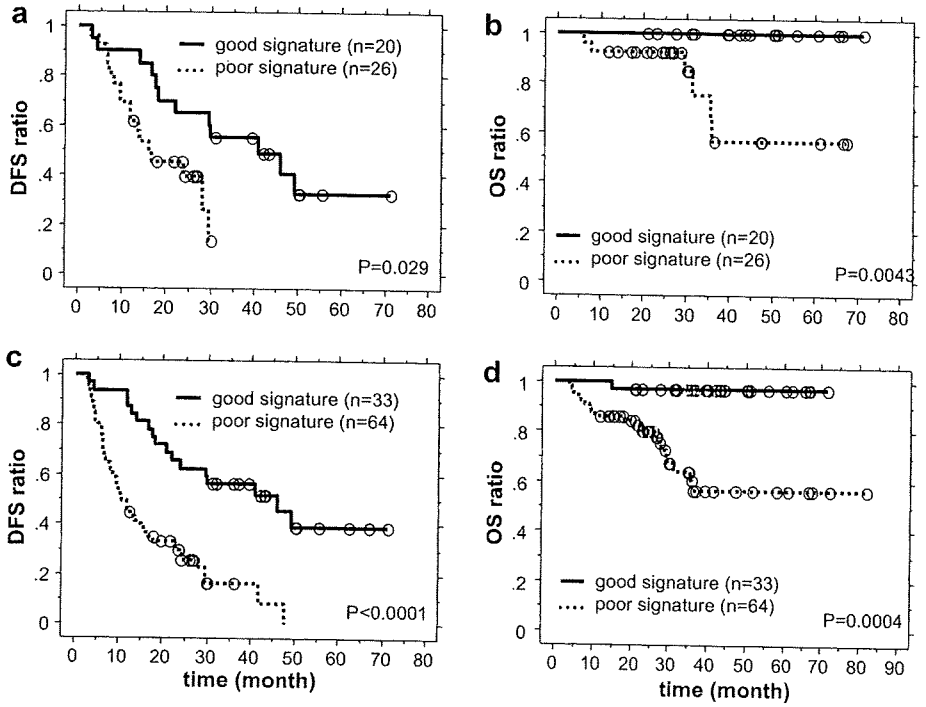

Fig. 3 – Disease-free survival curves and overall survival curves calculated using the Kaplan–Meier method for the independent cases. (a) DFS curves and (b) OS curves of the independent group C (*n* = 46). (c) DFS curves and (d) OS curves of the entire group of independent cases (*n* = 97) composed of groups C and D. Differences in survival curves were estimated by the log-rank test.

the Student t-test. Survival curves were computed using the Kaplan–Meier method, and differences between survival curves were compared using the log-rank test. To evaluate the risk associated with the prognostic variables, the Cox model with determination of the hazard ratio was applied; a 95% confidence interval was adopted. Statistical analyses were conducted using the SPSS software (version 11.0.1 J, SPSS Inc., Chicago, IL). We also performed network analysis using the Ingenuity Pathways Analysis (Ingenuity systems, Mountain View, CA; http://www.ingenuity.com), a web-based application.

Table 2 – Univariate analysis of the independent group C.

Parameter	Independent group C (n = 46)	P Value*	
		DFS	OS
Sex			
M	37	0.147	0.878
F	9		
Age, years			
<65	24	0.781	0.589
⩾65	22		
HB infection			
–ve	26	0.791	0.776
+ve	20		
HC infection			
–ve	18	0.467	0.980
+ve	28		
PIVKA-II			
<45 AU/ml	36	0.646	0.170
⩾45 AU/ml	10		
Capsule formation			
–ve	13	0.199	0.942
+ve	33		
Edmondson Grade			
I/II	27	0.479	0.479
III/IV	19		
CLIP score			
0–1	44	0.874	0.141
2–	2		
Liver status			
Child A	38	0.920	0.530
Child B	8		
AFP			
<400 ng/ml	38	0.724	0.374
⩾400 ng/ml	8		
Tumour diameter			
<5 cm	46	–	–
⩾5 cm	0		
Vascular invasion			
–ve	46	–	–
+ve	0		
Tumour multiplicity			
Single	46	–	–
Multiple	0		
Molecular-based diagnosis			
Poor prognosis	26	0.029	0.0043
Good prognosis	22		
Follow-up, months (median)	30 (4–81)		

* P Value was calculated by log-rank test according to the result of molecular diagnosis for DFS time.

Table 3 – Univariate analysis of the entire group of independent cases of independent groups C and D.

Parameter	Entire group (n = 97)	P Value*	
		DFS	OS
Sex			
M	80	0.604	0.582
F	17		
Age, years			
<65	42	0.892	0.850
⩾65	55		
HB infection			
–ve	51	0.584	0.416
+ve	46		
HC infection			
–ve	39	0.963	0.653
+ve	58		
PIVKA-II			
<45 AU/ml	73	0.897	0.387
⩾45 AU/ml	24		
Capsule formation			
–ve	19	0.730	0.748
+ve	78		
Edmondson grade			
I/II	49	0.015	0.169
III/IV	48		
CLIP score			
0–1	69	0.009	0.0024
2–	28		
Liver status			
Child A	81	0.229	0.032
Child B	16		
AFP			
<400 ng/ml	63	0.103	0.021
⩾400 ng/ml	34		
Tumour diameter			
<5 cm	72	0.062	0.021
⩾5 cm	25		
Vascular invasion			
–ve	82	0.187	0.0058
+ve	15		
Tumour multiplicity			
Single	65	0.0046	0.0033
Multiple	32		
Molecular-based diagnosis			
Poor prognosis	64	<0.0001	<0.0001
Good prognosis	33		
Follow-up, months (median)	30 (4–81)		

* P Value was calculated by log-rank test according to the result of molecular diagnosis for DFS time.

3. Results

3.1. *Differentially regulated genes during the training phase*

In the training phase, we examined the accuracy of prediction of HCC recurrence using full genes based on a WV algorithm with a leave-one-out cross validation approach. The accuracy of each gene set is shown in Fig. 2. The gene set of 0.0004% of P value using permutation test with 10,000 random trials marked the highest accuracy. We defined these differentially expressed 172 genes (P = 0.0004%) as the informative gene set. Supplementary Table 1 provides a list of the informative genes. The results of molecular-based diagnosis system were correct in 34 of 42 cases. When compared with each annotated group, this system correctly classified 18 of 21 cases with poor prognosis and 16 of 21 cases with good prognosis in this set.

3.2. *Results of molecular diagnosis of the independent group C*

We adopted the prediction system constructed during the training phase to the independent group C, and compared DFS and overall survival (OS) of the patients between the two diagnosis groups (Fig. 3A). Both the DFS and OS ratios were significantly lower in patients diagnosed as 'poor signature'. The DFS curves showed significant difference between the two groups (log-rank test: P = 0.029) and all the seven patients who died of cancer were diagnosed as poor signature (P = 0.0043) (Fig. 3B). To compare other clinicopathological indicators with DFS and OS, we performed univariate analysis. Only molecular diagnosis was significantly different (Table 2).

3.3. *Results of molecular diagnosis of independent group D*

For cases of the independent group D, the DFS ratio and OS ratio were significantly lower in cases diagnosed as 'poor signature'. The log-rank test indicated that the DFS ratio (P = 0.0011) and OS ratio (P = 0.035) were significantly different between the 'poor signature' and 'good signature' groups (Figure not shown).

3.4. *Results of whole independent cases and evaluation of prediction ability of molecular diagnosis relative to other conventional poor prognostic indicators*

Our prediction system was further tested in the entire group of 97 cases (groups C and D). These cases were divided into 64 cases with poor signature and 33 cases with good signature based on the prediction system. Fig. 3C and D show the DFS and OS curves, respectively, for the two groups, according to the results of the prediction system. Kaplan–Meier survival estimates showed that DFS ratio was significantly lower in cases diagnosed as 'poor signature' than in patients diagnosed as 'good signature' (P < 0.0001). Twenty of 21 patients who died of cancer were of the 'poor signature' group and their OS curves were statistically different (P = 0.0004).

To compare our molecular prediction system with other conventional clinicopathological indicators, we performed univariate and multivariate analyses for DFS and OS. Univariate analysis of each factor for DFS time showed nearly significant differences with regard to Edmondson grade, AFP, tumour diameter, vascular invasion, tumour multiplicity and the result of molecular diagnosis (Table 3). To test the independence of the molecular prediction system, we performed multivariate Cox analysis. The result of the molecular prediction system was an independent factor (P < 0.0001), with a hazard ratio of 3.29 (95% CI 1.83–5.91) for the DFS ratio (Table 4). As for the OS ratio, the result of the molecular prediction system was also an independent factor (P = 0.013), with a hazard ratio of 13.28 (95% CI 1.72–102.63) (Table 4).

4. Discussion

The major finding of the present study was that early intrahepatic recurrence in patients who had undergone curative resection of HCC can be predicted accurately using our anal-

Table 4 – Results of multivariate analysis of the entire group of independent cases.

Variables	Hazard ratio	95% CI	P Value
Multivariate analysis of the entire group of independent cases (n = 97, DFS)			
Molecular diagnosis: poor signature (*versus* good signature)	3.29	1.83–5.91	<0.0001
Tumour multiplicity: multiple (*versus* single)	2.21	1.34–3.65	0.002
Edmondson grade: III/IV (*versus* I/II)	1.88	1.11–3.18	0.018
Tumour diameter: ⩾5 cm (*versus* <5 cm)	1.40	0.78–2.52	0.26
AFP: ⩾400 ng/ml (*versus* <400 ng/ml)	1.17	0.60–2.20	0.62
Vascular invasion: +ve (*versus* –ve)	0.93	0.46–1.87	0.84
Multivariate analysis of the entire group of independent cases (n = 97, OS)			
Molecular diagnosis: poor signature (*versus* good signature)	13.28	1.72–102.63	0.013
Tumour multiplicity: multiple (*versus* single)	3.06	1.16–8.06	0.024
Liver status: Child B (*versus* Child A)	2.38	0.90–6.29	0.08
Vascular invasion: +ve (*versus* –ve)	2.20	0.73–6.67	0.16
Tumour diameter: ⩾5 cm (*versus* <5 cm)	2.02	0.67–6.05	0.21
AFP: ⩾400 ng/ml (*versus* <400 ng/ml)	1.52	0.48–4.83	0.47
Edmondson grade: III/IV (*versus* I/II)	0.97	0.35–2.71	0.96

ysis system of gene expression patterns. Characteristic genes were selected by comparing the gene expression pattern between cases with multiple intrahepatic recurrences within 2 years and cases without recurrence over 3 years during the system training phase. The molecular prediction system accurately detected the high-risk group for early recurrence. Multivariate analysis identified molecular diagnosis, tumour multiplicity, and Edmondson grade as the independent factors. Taking into consideration that the majority of the patients who undergo curative resection become negative for the conventional poor prognostic indicators, molecular diagnosis could be potentially useful clinically for detecting patients at high-risk for early recurrence.

To improve the predictive accuracy, it is essential to clear the criteria of a homogeneous training set.[23] Our definition of the two groups was based on a study reported on the analysis of DFS ratio in HCC patients.[24] The DFS curve is composed of two regression lines. The majority of patients who developed recurrence within 2 years and who formed the first regression line were considered to have poor prognosis. On the other hand, the recurrence ratio of patients who showed no recurrence over a 3-year follow-up was almost the same as the annual relapse ratio of HCC in patients with hepatitis and their prognosis was better. This constant decrease in DFS ratio in the late recurrence cases is not usually observed in hepatectomised patients with liver metastasis from intestinal cancer.[26,27]

Recurrence of HCC is based on residual intrahepatic recurrence (IM) or multicentric metastasis (MC). IM is thought to originate from the primary cancer, while MC is considered to reflect a significant influence of the underlying liver status.[27,28] The two recurrence patterns are clinically important in patients with HCC where intrahepatic metastatic spread carries in general a poorer prognosis than that with multicentric nodules.[24] However, the conventional approach of histopathological examination is limited with regard to the differentiation of recurrence patterns as IM or MC.[29] With regard to the results of the validation phase, 17 patients survived for more than 3 years and only three of these 17 were diagnosed as poor signature and one of three cases was considered to have recurrence by metastasis from the primary tumour. On the other hand, 59 of 97 patients had intrahepatic recurrence within 2 years. This prediction system diagnosed these samples into 46 cases of poor signature and 13 cases of good signature. All 13 patients did not undergo a repeat resection, and thus pathological examination of recurrence pattern could not be conducted. However, as for the overall survival time in these 13 patients, only one died of cancer at 14 months postoperatively, while the remaining 12 patients remain alive for more than 21 months after surgery (range 21–48 months, median: 35 month). About half of the 13 patients had long survival though they had early recurrence. When we consider the relationship between study design and these results, the two groups diagnosed by our molecular-based diagnosis system may represent two recurrence patterns. The poor signature group may represent cases with recurrence due to IM, and the good signature group may represent cases with recurrence due to MC. This study may be clinically meaningful and helpful to solve the mechanism of recurrence patterns.

The prognoses of 42 patients during the training phase were polarised and those of the remaining 46 of the independent cases were intermediate. The 2-year survival ratio of the good signature independent cases was 65%, which was not as good as the annual relapse ratio of HCC. However, it is meaningful that the independent group C without any poor prognostic indicators could be divided into two groups of different prognoses. The reason for the discrepancy between the DFS ratio of cases diagnosed as good signature and annual relapse ratio is probably due to the fact that the independent group C did not include cases of extremely poor prognosis with early fulminant recurrence or cases of extremely good prognosis without long-term intrahepatic recurrence. Further analysis of cases with natural distribution of clinical status may help in moving the result of cases with good signature towards the annual relapse ratio.

In the conventional theory of metastasis, it is thought that tumours acquire the metastatic potential based on their progression and that metastasis occurs in the late phase. Based on this theory, recurrence could not be predicted by the analysis of the primary tumour. This theory was challenged recently by a new paradigm, which argues that the metastatic potential is not acquired in proportion to cancer progression but is already encoded in the primary tumour. Ramaswamy and colleagues[30] reported that a gene expression programme peculiar to metastasis may already be present in the bulk of some primary tumours and that a predictive diagnosis for metastasis was possible based on the analysis of the primary tumour profile. Several studies suggested that the molecular programme of primary tumour is generally retained in its metastasis.[31–33] Interestingly, Hoshida and colleagues[34] reported that the gene expression profiles in early-stage HCC tumours were highly associated with late recurrence (more than 2 years after resection) in the surrounding non-tumoural liver tissue but not in the tumoural tissue, indicating that environmental exposure leads to an increased potential of future malignant transformation. In this study, we evaluated the predictability of early recurrence using gene expression profiles of whole tumour tissue, based on the assumption that IM related to early recurrence might originate from the primary cancer.[27,28] For the entire group of independent cases, 78% of the recurrent cases within 2 years were diagnosed as poor signature. Some metastatic events may occur according to tumour progression, but cases with metastasis via the new paradigm should exist. Application of the theory of this paradigm may lead to the design of new diagnostic methods for cases in whom conventional clinicopathological parameters could not predict the prognosis.

Among the informative gene set, various genes correlate with cancer progression and carcinogenesis. PPARBP is regulated by RB18A and acts as a transcription cofactor by regulating the activity of p53wt transactivation on physiological promoters. Furthermore, downregulation of RB18A results in p53wt-dependent apoptosis.[35] RREB-1, a novel zinc finger protein, is involved in the differentiation response to Ras.[36] The Ras family is thought to be particularly important determinant of tumour initiation and progression.[37] BCL2 is one of the well-known tumour suppressor genes and is associated with recurrence and survival of HCC patients.[38,39] HDAC1 is reported to induce hyperacetylation of nucleosomal histones