

Figure 3: Donor species-specific FIX mRNA expressions in uPA/SCID mouse livers transplanted with either canine or human hepatocytes. A) Canine factor IX (FIX) RNA copy numbers relative to canine GAPDH (reflecting RNA copy numbers per canine hepatocyte), based on plasma cFIX:Ag levels. (Low, <40%; Medium, 41–80%; High, >81% of normal canine plasma. n=4, 4, and 5, respectively). N.C.: negative control: non-transplanted uPA/SCID mouse livers (n=4). P.C.: positive control: normal beagle dog livers (n=3). B) Human

FIX RNA copy numbers relative to human GAPDH (reflecting RNA copy numbers per human hepatocyte), based on the repopulation rate (R.R.) estimated from human albumin concentrations as described in *Materials and methods*. (Low, <40%; Medium, 41–65%; High, >66%. n=4, 4, and 4 respectively). N.C.: negative control: non-transplanted uPA/SCID mouse livers (n=4); P.C.: positive control: normal human liver tissues (n=3).

We also measured canine albumin levels in the plasma of several uPA/SCID mice that received hepatocytes from the seven-year-old beagle, and demonstrated a highly significant correlation between the canine albumin and canine FIX:Ag levels ($R^2=0.9963$) (Fig. 1D). Assuming that the plasma concentrations of albumin and FIX:Ag in normal dogs are 5 g/dl and 5,000 ng/ml, respectively, the weight ratio of albumin to FIX:Ag in normal canine plasma was calculated to be 10,000:1. These data suggested that the synthesis of canine FIX and albumin in the transplanted animals was similar to that of normal canine liver (i.e. 15% FIX:Ag of normal canine plasma corresponds to 750 ng/ml, and the ratio of 0.6 g/dl to 750 ng/ml approximates to 10,000:1). Immunohistochemical staining for canine albumin in sections obtained at day 55 after transplantation demonstrated a large area of the liver was positive in the recipients with high plasma canine FIX:Ag (33.2%) (Fig. 2B-C), whereas only a small area of liver was positive in mice with low plasma FIX:Ag levels (2.0%) (Fig. 2A). Histological examination of serial liver sections revealed that the canine albumin-positive area was composed of morphologically normal hepatocytes (Fig. 2D-E) indicating that the normal canine hepatocytes had progressively propagated in the uPA/SCID livers.

The uPA/SCID mice that received canine hepatocytes were divided into three groups according to their plasma canine FIX:Ag levels (low <40%, medium 41–80%, and high >81%). mRNA levels of canine FIX were normalized using canine GAPDH mRNA measurements (FIX / GAPDH). As shown in Figure 3A, canine FIX / canine GAPDH expression was similar in the three groups with no statistically significant difference. This suggests that canine hepatocytes proliferated within the uPA/SCID livers without reducing the steady-state levels of canine FIX gene expression and/or degradation. We confirmed that RNA samples from untreated uPA/SCID livers were not amplified by the primer set used for canine FIX and GAPDH detection (Fig. 3A).

Proliferation of FIX-producing human hepatocytes in uPA/SCID mouse livers

Human hepatocytes were transplanted into the liver of uPA/SCID mice (n=12). The proliferation and propagation status of the transplanted hepatocytes were assessed by periodic measurement of human albumin levels in the recipient blood, and the repopulation rate of human hepatocytes in the uPA/SCID livers was assessed as described previously (35, 38). Human FIX:Ag was detected in the plasma of 75% of the mice (9 out of 12) between 67 and 84 days after transplantation, and the circulating plasma levels ranged between 6–58% found in normal humans. The results from our study demonstrated that the FIX:Ag levels were highly correlated with the human albumin levels ($R^2=0.8714$) (Fig. 4A). To examine the biological function of the secreted human FIX, we compared the repopulation rate with FIX:C assays (Fig. 4B). Although both murine and human FIX:C could be measured using the clotting assay, we were able to estimate the approximate levels of the *de novo* expressed human FIX:C present in our samples. Plasma levels of FIX:C in untreated uPA/SCID mice were less than 50% of the levels normally found in human plasma, and we expected to increase the FIX:C levels up to 100% following the humanization of the murine livers. Similar to the canine transplants, the results showed that mice with a high repopulation rate had higher FIX:C than those with low repopulation rates ($R^2=0.7245$). These data suggested that secreted human FIX protein was biologically active. To clarify the proliferation status of transplanted human hepatocytes in uPA/SCID mouse liver, we also transplanted human hepatocytes isolated from another human subject (a 2-year-old Caucasian male) into a new set of uPA/SCID mice (n=9). Using these mice, we measured plasma human FIX:Ag levels, total plasma FIX:C levels, and human plasma albumin concentrations from samples obtained periodically from the recipient mice during an eight-week period after transplantation. As shown in Fig-

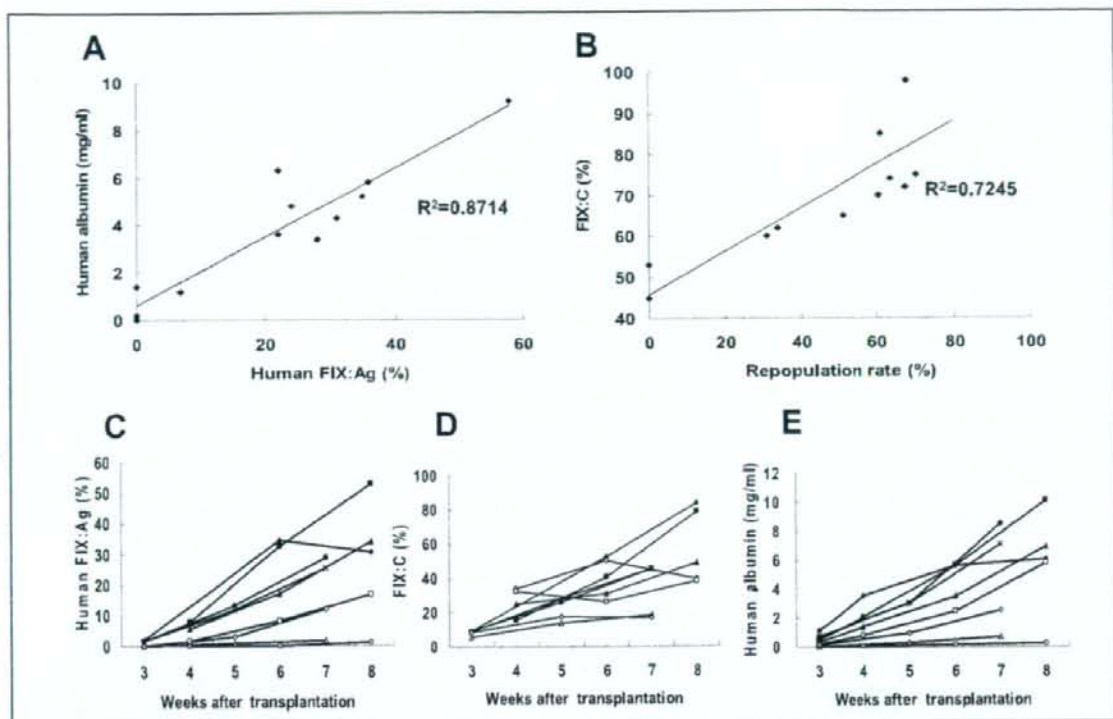


Figure 4: Proliferation of transplanted human hepatocytes in uPA/SCID mouse livers assessed by recipient blood analyses. A) Relationship between blood human albumin and plasma human factor IX (FIX) antigen (hFIX:Ag) concentrations of uPA/SCID mice transplanted with human hepatocytes ($n=12$). B) Relationship between total plasma FIX coagulation activity (FIX:C; reflecting both murine and human FIX activities) (% of normal human plasma) in uPA/SCID mice transplanted with human hepatocytes based on the repopulation rate

estimated from human albumin concentrations as described in *Materials and methods*. The recipient blood samples used for these assays were obtained 67–84 days after transplantation. C–E) Time course of plasma hFIX:Ag levels (C), total plasma FIX:C levels (D), and blood human albumin levels (E) of the recipient mice after human hepatocyte transplantation ($n=9$) (different set of experimental data from that shown in A and B).

ure 4C–E, each of our measured parameters were found to be increased after transplantation in most of the mice, which was indicative of a robust proliferative status of the transplanted human hepatocytes.

Liver sections obtained from mice with high plasma human FIX:Ag levels were found to have strong positive staining in the hepatocytes for human FIX as determined by immunohistochemistry (Fig. 5B). In marked contrast, only a small portion of the liver stained positive in sections of recipient mice that were detected to have low (i.e. <1%) circulating levels of plasma FIX:Ag level (Fig. 5A). These results were consistent with the findings that the *de novo* production of haemostatically active human FIX in the circulation was dependent on the viability and persistence of the transplanted hepatocytes in the recipient uPA/SCID livers.

The uPA SCID mice that received human hepatocytes were divided into three groups according to the repopulation rate (low <40%, medium 41–65%, and high >66%). Human FIX mRNA levels were normalized using glyceraldehyde-3-phosphate dehy-

drogenase (GAPDH) mRNA measurements (FIX:GAPDH). As shown in Figure 3B, the ratio of human FIX:GAPDH mRNA expression in the recipient livers was not significantly different among the three groups. We further confirmed that extracted total RNA from untreated uPA/SCID livers were not amplified by the primer set used for human FIX and human GAPDH detection (Fig. 3B), demonstrating the specificity of the primers to human and not murine FIX expression. Similar findings were determined in the canine hepatocyte transplantation experiments in which we confirmed that human hepatocytes proliferated in uPA/SCID mouse livers and retained their ability for transcribing the human FIX gene.

It has been reported that differentiated liver function (e.g. mRNA expression of albumin) may be suppressed when hepatocytes are subjected to various proliferative stimuli (40, 41). It is not known, however, if hepatocyte proliferation could directly influence FIX mRNA expression. To address this question, we compared FIX mRNA expression in quiescent and proliferating mouse livers. Liver proliferation was induced by performing a

70% hepatectomy in C57/BL6 wild-type mice, and the remnant liver lobes were subsequently harvested two days after the hepatectomy, which is the time point where hepatocyte proliferation is at its peak. The quiescent (non-proliferative) liver samples used in these experiments were the resected liver lobes obtained from the same mice in which the partial hepatectomy was performed. The relative FIX mRNA expression (FIX:GAPDH) was ~35% lower in the proliferating liver compared to the quiescent liver ($p=0.029$; Fig. 6). These results would suggest that the proliferative status of the transplanted hepatocytes may affect the production of FIX, and the reason for the lower FIX mRNA expression found in the recipient mice compared to the control livers as shown in Figure 3 may be due to active proliferation by the transplanted hepatocytes. If the FIX mRNA expression levels found in the normal canine and human livers were recalculated to account for a 35% reduction in response to proliferative stimuli, the significant differences between the four groups shown in Figure 3 became non-significant (data not shown). This suggests that proliferating hepatocytes in uPA/SCID mice have the capability of expressing normal levels of canine and human FIX following transplantation.

Discussion

We have established an *in vivo* system to propagate human and canine hepatocytes in uPA/SCID mouse livers, and these transplanted hepatocytes are capable of retaining their cellular machinery to produce coagulation FIX. The capabilities of these propagating transplanted hepatocytes to synthesize FIX were confirmed by the expression of FIX mRNA, FIX-protein production and secretion, and its coagulation activity. The main reason we decided to study canine hepatocyte in addition to human hepatocytes for transplantation, is the availability of a pre-clinical large animal model for haemophilia B studies (42), which will be able to be used for proof-of-concept experiments.

Hepatocytes are the only cells that are known to synthesize FIX (18), and successful liver transplantation has resulted in restoration of normal coagulative properties in patients with haemophilia B (17). Although there are obvious benefits in surgically transplanting whole livers in haemophilic patients with critical life-threatening liver diseases such as chronic active hepatitis, this approach is likely not appropriate for most patients due to other obvious negative risks associated with this type of procedure. For this reason, the hepatocyte transplantation approach described in the current study, which is less invasive and requires fewer donor livers, may provide a viable alternative strategy to organ transplantation. Recent trials have highlighted successful application of hepatocyte transplantation in two patients with coagulation factor VII deficiency (31, 43). Following hepatocyte transplantation, both patients were found to have achieved significant and prolonged therapeutic benefit with a marked decrease in the infusion of exogenous recombinant factor VIIa due to episodic bleeding (44, 45).

We demonstrated that canine and human hepatocytes progressively proliferated and propagated in the recipient livers of uPA/SCID mice. Real-time PCR analysis at various stages of hepatocyte propagation showed that FIX mRNA expression per transplanted cell (per donor-specific GAPDH mRNA level) was

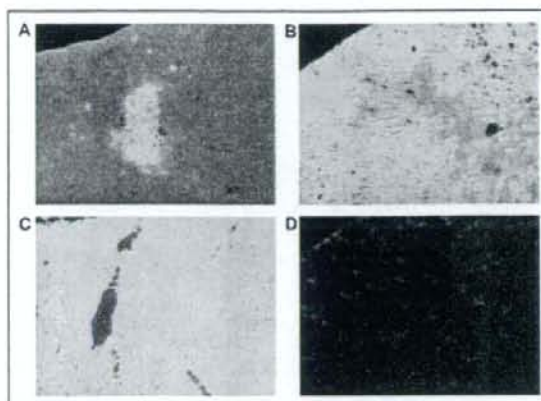


Figure 5: Mouse liver chimerism with proliferated human hepatocytes. Immunofluorescent staining of human FIX in liver sections of uPA/SCID mice after human hepatocyte transplantation. Liver sections of mice with <1% plasma hFIX:Ag level (A) and mice with 34% hFIX:Ag plasma level (B). Red-stained area indicates proliferated human hepatocytes producing hFIX. Positive control (normal human liver tissue) (C) and negative control (non-transplanted uPA/SCID mouse liver) (D) indicate the staining specificity for human FIX. Original magnification, $\times 100$.

stably maintained for the duration of the experiment. Plasma FIX:Ag levels were highly correlated with the propagation status of the transplanted hepatocytes as determined by the blood levels of canine and human albumin. Furthermore, the procoagulant function of the secreted canine and human FIX was confirmed by clotting assays. FIX:C increased from baseline levels (less than 50% of normal human plasma) to normal human or canine FIX:C levels (about 100% and 200%, respectively) as repopu-

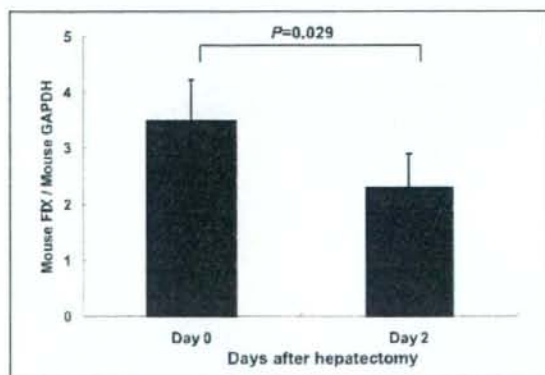


Figure 6: Comparison of factor IX (FIX) mRNA expression levels in quiescent and proliferating livers. Day 0 liver samples (quiescent status) were obtained from C57/BL6 mice at the time of 70% partial hepatectomy ($n=6$). The remnant liver lobes of the mice were harvested at day 2 were used for the assessment for proliferating status ($n=6$). Relative FIX mRNA expression was expressed as murine FIX / murine GAPDH.

lation by transplanted hepatocytes progressed. The data from our experiments indicated that post-transcriptional modification of FIX, including cleavage and removal of the pre-pro leader sequence of 46 amino-acids, γ -carboxylation of the first 12 glutamic acid residues, and partial β -hydroxylation of Asp 64 (46), must have occurred within the transplanted hepatocytes to maintain biologically active haemostatic function.

Hepatocytes from a one-year-old dog demonstrated high proliferation activity compared with cells from an older (7-year-old) dog as evidenced by the more rapid increase and its higher sustained levels of plasma canine FIX:Ag. These results are consistent with earlier findings by our group in which we reported that human hepatocytes from a younger donor occupied a larger proportion in the recipient uPA/SCID mouse liver compared with transplanted hepatocytes from an older donor (47). One possible reason for the enhanced growth potential of hepatocytes in these circumstances may be due to an elevated expression of cell cycle proteins in hepatocytes from younger compared to older donors (48). With the present study as well as previous work in the literature, we believe that the age of the donor makes a difference on the proliferation and repopulation of the transplanted hepatocytes in uPA/SCID mouse livers.

Human hepatocytes propagated in uPA/SCID mice could be isolated and purified using cell-sorting technology (38). Recently, our group has developed a procedure to isolate human hepatocytes that were propagated in uPA/SCID mouse livers, and these isolated hepatocytes were confirmed to be biologically functional compared to original primary hepatocytes, demonstrating the expression of cytochrome P450 (CYP) (38). We have also been experimentally successful in engineering functional liver tissue using isolated hepatocytes transplanted under the kidney capsule or in the subcutaneous space by demonstrating coagulation factor VIII expression (25–30). More recently, Azuma et al. (49) reported an alternate method to propagate human hepatocytes in living mice that furthers the utility of hepatocyte transplantation. Based on these developments, propagated human hepatocytes with FIX expressions should become a valuable cell source in establishing novel cell-based therapies for direct transplantation or development of tissue engineering strategies in the treatment of haemophilia B.

For the eventual translation of cell-based therapies using the propagated human hepatocytes for haemophilia B to be successful in the clinics, several potential obstacles will need to be considered and overcome. First, contamination of murine cells during the isolation of the transplanted human hepatocytes must be

minimized. Second, increasing the engraftment rate of the transplanted hepatocytes into the recipient liver. Lastly, the survival and viability of the transplanted allergenic hepatocytes must be prolonged. With regards to the first issue, the contaminating murine hepatocytes during the isolation of human cells from the mouse liver could be overcome by utilizing recipient transgenic mice that have been incorporated with a inducible suicide gene. In the presence of the inducing agent, the murine cells would be preferentially eliminated and increase the purity of the human hepatocyte mixture leading to enhanced clinical safety. To overcome the low engraftment rate found in the current and previous studies, the recipient livers will require some type of preconditioning regimen to maximize the efficiency and engraftment. Slehria et al. (50) reported an effective and non-invasive pre-treatment protocol in which the administration of phentolamine, an adrenergic receptor blocker, resulted in the dilation of the hepatic sinusoidal vasculature leading to enhanced hepatocyte engraftment rate. For the last issue regarding the limited graft survivability of the donor cells due to the activation of the host immune system, it will be important to design an immunosuppressive regimen specific for hepatocyte transplantation and monitoring systems for the early rejection need be established. These issues will need to be studied and overcome to substantiate the utility of this approach for the treatment of haemophilia and other congenital liver disorders.

In all, the present study has demonstrated the utility of hepatocyte transplantation for the therapeutic production of coagulation factor IX. As we continue to overcome the obstacles associated with this approach, this transplantation methodology will evolve into a novel approach to treat not only liver diseases associated with haemophilia but other forms of congenital liver diseases.

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◆ 原 著 ◆

軽症血友病 A から検出される第 VIII 因子 R531H 変異の 第 VIII 因子活性とその特徴

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日本人軽症血友病 A 患者 2 名の第 VIII 因子遺伝子解析から病因遺伝子異常として Arg531His 変異を同定した。患者は共に軽症のフェノタイプであった。両者の第 VIII 因子は、比活性は同等であるものの、活性および抗原のレベルはかなり異なっていた。患者 1 の血漿の第 VIII 因子活性を 14 種類の APTT 試薬を用いて凝固 1 段法で測定した結果、25.2% から 48.1% の範囲で測定され、用いる APTT 試薬によって大きく異なっていた。この測定値の乖離は、APTT 試薬の pH との間に負の相関関係が認められた。患者 2 の血漿をサンプルとした検討から、検体の希釈にイミダゾール緩衝液を用いることで乖離を最小化できる可能性が示唆された。これらのことから Arg531His 変異の第 VIII 因子レベルは、患者によって異なっており、またさらに測定法によっても大きく影響を受けることが明らかとなった。Arg531His のような軽症例を的確に検出・把握できる標準的な検査法を確立する必要がある。

Key words: Mild hemophilia A, Mutation, One-stage clotting assay, Discrepancy

緒 言

先天性血友病 A は第 VIII 因子 (FVIII) をコードする第 VIII 因子遺伝子 (F8) の異常によって引き起こされ、X 染色体性劣性の遺伝形式をとる。本疾患の呈する出血症状は血中に存在する FVIII 活性 (FVIII:C) と相関することから、その重症度は FVIII:C の値により 3 グループ (重症、中等症、軽症) に分類される。軽症血友病 A は 5% を上回る FVIII:C を有する場合であり、血友病 A の約 2 割がこれに分類される¹⁾。

F8 は Xq28 に位置する全長 186 kb の大きな遺伝子であり、1984 年にクローニングされた²⁾。血友病 A 患者の F8 解析により、これまでに血友病 A を引き起こす様々な遺伝子異常が

同定されてきたが、軽症例についてはその責任遺伝子異常は点変異によるミスセンス変異に限定されるといっても過言ではない。また軽症例から検出されるミスセンス変異は多くが分子異常症であり、FVIII:C の構造と機能の解析には極めて重要な知見をもたらす。

FVIII:C は凝固 1 段法、凝固 2 段法、合成基質法の 3 つの方法で測定されるが、一部の軽症では測定法によって異なった FVIII:C を呈することが報告された³⁾。立体構造の解析から、これらの変異部位の多くは 3 つの A サブユニットのインターフェイスに位置することが確認され、FVIII 分子からの A2 サブユニットの解離の動態が、測定法ごとに異なる FVIII:C に関与することが明らかにされた^{4) 5)}。A2 サブ

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ユニット内の Arg531His (R531H) 変異は, A1 サブユニットとのインターフェイスに位置し, 測定法によって異なる FVIII:C を呈する⁴⁾.

今回我々は軽症血友病 A 症例の F8 解析から検出した 2 例の R531H を対象に, その特徴について検討した.

研究対象, 材料および方法

対象

症例 1 は 61 歳の男性. 手術の準備のため, 以前, 凝固検査の異常を指摘されていたため精査を目的に近医を受診した. これまでに陸上競技後の膝関節での出血と, 扁桃を傷つけた際の止血困難がそれぞれ一度ずつあるのみで, 日常生活の中で出血症状を認めないが, 母方には出血傾向を有する男性が複数存在する. 凝血学的検査では APTT に延長を認めなかった. 因子定量の結果では唯一 FVIII が約 60% に低下していたことから血友病 A が疑われたが, 凝血学的検査のみでは血友病 A の診断には至らず東京医科大学病院にて遺伝子解析を施行した.

症例 2 は 30 歳の男性. 抜歯時の止血困難にて 14 歳時に血友病 A と診断される. 幼少時から鼻出血が多く, 激しい運動 (サッカー) の後に前下腿部に血腫ができた経験もあったが, 日常生活において問題となるような出血症状は認めないため, 血液製剤の投与を受けることもなかった.

凝血学的解析

①活性化部分トロンボプラスチン時間 (APTT)

APTT は自動凝固測定装置 ACL-9000 (Instrumentation Laboratory 社, IL 社) を用いて測定した. 通常の APTT 試薬には HemosIL™ APTT-SP (IL 社) を, サンプル血漿の希釈液にはファクターゲイリューエント (IL 社) を用いた. また, コントロール血漿には凝固因子アッセイコントロール血漿 (GK-FACT: ジョージキングバイオメディカ

ル社) を用いた.

② FVIII:C 測定 (凝固 1 段法)

凝固 1 段法による FVIII:C 測定は前述の APTT を用いた. 第 VIII 因子欠乏血漿には GK-08 (ジョージキングバイオメディカル社) を用いた.

③凝固 1 段法における APTT 試薬の影響の検討

患者 1 血漿の FVIII:C を入手可能であった合計 14 種類 (我々が日常用いている HemosIL™ APTT-SP を含む, 表 1) の APTT 試薬を用いて測定し, 検討した. 対照として FVIII 欠乏血漿と凝固因子アッセイコントロール血漿を 6:4 で混合して作製した FVIII:C 40% のコントロール血漿を測定し, 患者 1 血漿の成績と比較した. また, 用いた各 APTT 試薬については構成成分 (活性化合物, リン脂質) を添付文章から調べ (表 1), pH を測定し, FVIII:C 測定との関連について検討した.

④凝固 1 段法における試薬 pH の影響の検討

我々が通常用いている HemosIL™ APTT-SP (pH 7.54) の pH を, 塩酸または水酸化ナトリウム溶液を用いて 6.96, 7.17, 7.33, 7.71 に変え, 試薬 pH の FVIII:C 測定に与える影響を検討した. 患者 2 血漿および, FVIII 欠乏血漿と凝固因子アッセイコントロール血漿を 8:2 で混合した FVIII:C 約 20% のコントロール血漿を測定し比較した.

⑤凝固 1 段法における乖離の最小化を目的とした検体希釈液の検討

我々が通常用いている HemosIL™ APTT-SP と, R531H 症例の FVIII:C 測定上 HemosIL™ APTT-SP とは明らかに乖離した測定値を精密に再現することのできたトロンボチェック APTT (S) を用いて乖離の最小化についての検討を行った. 患者 2 血漿と, FVIII 欠乏血漿と凝固因子アッセイコントロール血漿を 8:2 で混合した FVIII:C 約 20% のコントロール血漿を対照として測定した. 希釈液には我々が通常用いているファクター

表1 APTT reagents used in this study

No.	Reagent	Phospholipids	Activator	pH
1	PTT-reagent (Diagnostica Stago)	Cepharin	Kaolin	7.45
2	Actin FS (Dade Behring)	Cephalin (soybean)	Ellagic acid	6.72
3	Automated APTT (bioMerieux)	Cephalin (rabbit brain)	Silica	7.47
4	Thrombocheck APTT (Sysmex)	Cephalin (rabbit)	Ellagic acid	7.24
5	Platelin LS (bioMerieux)	Phospholipids (egg, bovine brain)	Silica	7.54
6	STA Cephascreen (Diagnostica Stago)	Cephalin	Polyphenols	7.61
7	Platelin L (bioMerieux)	Phospholipids (egg, bovine brain)	Silica	7.54
8	HemosIL SynthASil (Instrumentation Laboratory)	Synthetic phospholipids	Colloidal silica	7.23
9	STA APTT (Diagnostica Stago)	Cephalin	Celite	7.41
10	Actin (Dade Behring)	Cephalin (rabbit brain)	Ellagic acid	6.95
11	Thrombocheck APTT(S) (Sysmex)	Cephalin (soybean)	Ellagic acid	7.29
12	Pathromtin SL (Dade Behring)	Lecithin (soybean)	Silica	7.41
13	Cephotest (Axis-Shield)	Dried bovine brain extract	?	7.49
14	HemosIL APTT-SP (Instrumentation Laboratory)	Synthetic phospholipids	Colloidal silica	7.54

ダイリュエント (生理食塩水) 以外にペロナル緩衝液 (28 mM バルビタールナトリウム, 125 mM 塩化ナトリウム, pH 7.35), イミダゾール緩衝液 (50 mM イミダゾール, 100 mM 塩化ナトリウム, pH 7.3), トリス緩衝液 (20 mM トリス塩酸緩衝液, 150 mM 塩化ナトリウム, pH 7.4) を検討した。

⑤ FVIII:C 測定 (発色性合成基質法)

発色性合成基質法による FVIII:C 測定にはコATEST SP FVIII キット (Chromogenix 社) を用い、製造者の指示に従いマニュアル通りに測定した。

⑦ 第 VIII 因子抗原 (FVIII:Ag)

FVIII:Ag はアセラクロム VIII:Ag キット (Diagnostica Stago 社) を用い、製造者の指示に従いマニュアル通りに測定した。

8 遺伝子解析

F8 を構成する 26 のエクソンは、それぞれのイントロンと境界領域を含めて PCR にて増幅し、PCR 産物のダイレクトシーケンシングによって塩基配列を決定した。エクソン

14 は 3.1 kb と大きいことから、互いにオーバーラップする 8 つの領域に分けて解析した。本研究は東京医科大学医学倫理委員会の審査を経て、東京医科大学学長により承認されている『先天性血液凝固因子欠損 (欠乏) および異常症に関する遺伝子研究』に従い、患者には文章による説明と同意を取得したうえで遺伝子解析を行った。

成 績

1. 遺伝子解析結果

患者 1, 2 の F8 解析から R531H 変異が検出された。今回の研究において塩基配列を決定した F8 の他の領域には両者間に差異を認めなかった。

2. 各種測定法における FVIII:C 測定結果

両患者の FVIII:C (凝固 1 段法, 合成基質法) および FVIII:Ag を表 2 に示す。FVIII:Ag に比べ FVIII:C は著しく低値を示しており、こ

表 2 APTT and FVIII level of the two R531H patients

	APTT (cont. 32. 2)	FVIII : C		FVIII:Ag
		One-stage clotting assay	Chromogenic assay	
Patient 1	34.6sec	34.5%	32.9%	70.7%
Patient 2	48.3sec	19.9%	13.4%	48.1%

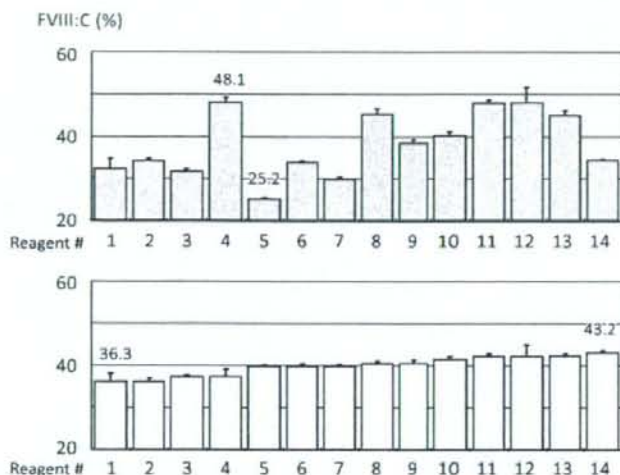


図 1 FVIII : C levels of R531H (patient 1) and 40% control plasma measured by 14 different APTT reagents.

The FVIII : C levels of patient 1 and 40% control plasma were measured by one-stage clotting assay with 14 different APTT reagents. The 40% control plasma showed similar values when assayed with each of the 14 reagents (range, 36.3%-43.2%) (lower graph). However, measurements of R531H differed with reagent (range, 25.2%-48.1%) (upper graph)

のことから R531H 変異は分子異常症であることが確認された。14 種類の APTT 試薬を用いての FVIII : C 測定では、40% FVIII : C コントロール血漿の測定値は APTT 試薬ごとに多少のばらつきが確認されるものの 40% を中心とした約 ±10% の範囲で測定されたのに対し、患者 1 血漿の測定値は最低 25.2% から最高 48.1% の広範囲で測定され、試薬ごとに著しい乖離が確認された (図 1)。

3. 乖離に影響する要因の解析

FVIII : C 乖離に影響する要因の解析のため、APTT 試薬の構成成分 (活性化物質, リン脂質) と pH の影響について検討した (表 1, 図 2)。FVIII : C は、活性化物質とリン脂質との間に何ら相関関係は認められなかった。しかしながら、pH は R531H の活性値に大きく関与し、pH7.2 ~ 7.4 で最も高値に測定される傾向があり、また pH が 7 未満であった 2 試薬を除いた 12 種類の APTT 試薬では負の相関関係 ($r = -0.748$ ($p = 0.005$)) が認められた。

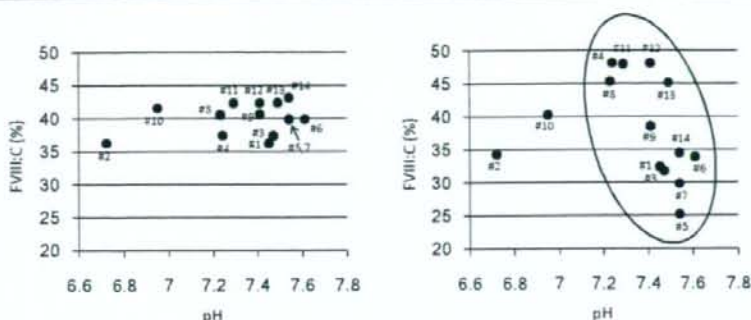


図2 Influence of APTT reagent pH on 40% control plasma and R531H (patient 1) FVIII:C measurement.

Excluding the reagent of the #2 and the #10, a negative correlation ($r = -0.748$) between pH and R531H measurement was observed (right graph).

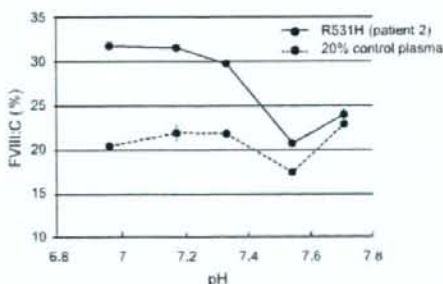


図3 Influence of APTT reagent pH on 20% control plasma and R531H (patient 2) FVIII:C measurement.

The FVIII:C levels of patient 2 and 20% control plasma were measured by one-stage clotting assay using different pH of HemosIL APTT-SP. Although pH of the APTT reagent similarly influenced FVIII:C measurement of both R531H and control plasma, it was more remarkable in the R531H.

4. 試薬 pH の影響の検討

HemosIL™ APTT-SP の pH (7.54) を、塩酸または水酸化ナトリウム溶液を用いて 6.96, 7.17, 7.33, 7.71 に変えて試薬 pH の FVIII:C 測定に与える影響を検討した。R531H, 20% FVIII:C コントロール血漿ともに、pH の変化により同様の測定値の変動がみられたが、R531H でのそれはより顕著であった (図 3)。

5. 乖離の最小化に関する検討

患者 2 の FVIII:C の乖離は希釈液に緩衝液を用いることで軽減し、イミダゾール緩衝液を用いた場合に最小であった (図 4)。

考 案

本論文では軽症血友病 A 症例の遺伝子解析から 2 例の血縁関係のない R531H 症例を検出し、その FVIII:C 測定における特徴について検討した。

R531H 変異は点変異のホットスポットである CpG ダイヌクレオチドでのグアニン (CGC) からアデニン (CAC) への置換によって引き起こされる。2007 年 8 月現在、血友病 A 変異データベース (The Haemophilia A Mutation, Structure, Test and Resource Site; HAMSTeRS, <http://europium.csc.mrc.ac.uk/usr/WWW/WebPages/main>) において同変異は 17 例の登録があり、点変異の 0.87% (17/1994)、軽症例の 2.21% (17/769) を占める。17 例の FVIII:C は 20~40% 程度での報告が多いが、最低 5% から最高 96% までの広範囲の値が記載されている。これまでに R531H 症例では、FVIII:C が凝固 1 段法と合成基質法間で乖離することが報告されており⁹⁾、また最近、凝固 1 段法でも、

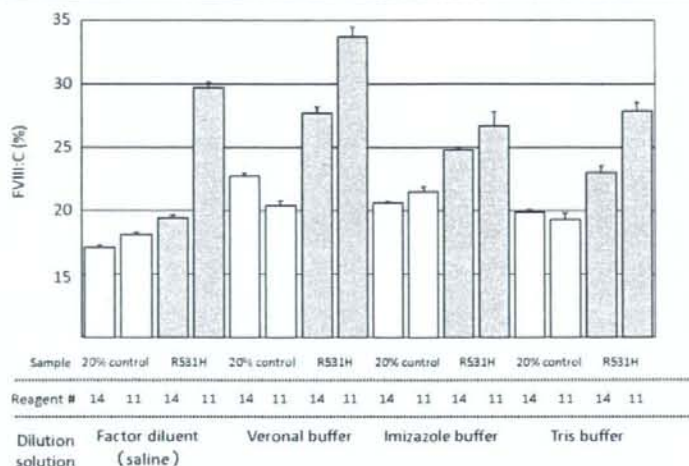


図 4 Influence of sample dilution solution on R531H (patient 2) FVIII : C measurement. The FVIII : C levels of patient 2 and 20% control plasma were measured by one-stage clotting assay with four different sample dilution solutions. Two APTT reagents (Reagents #11 and HemosIL™ APTT-SP) that showed high reproducibility under ordinary assay conditions were used. Differences in R531H FVIII:C measurements were reduced considerably with the use of imidazole buffer as the sample dilution solution.

一部の APTT 試薬間で FVIII : C に乖離が認められるとの報告がなされている¹⁰⁾。今回の患者 1 の解析では、我々が日常用いている APTT 試薬 (HemosIL™ APTT-SP) による測定値が、合成基質法による測定値と同程度となっていたため凝固 1 段法と合成基質法間で大きな乖離は認められなかったが、凝固 1 段法では APTT 試薬の違いによって大きな差異をもって測定された。これらのことから、FVIII : C 測定に関する情報がないことから断定はできないが、おそらくデータベースにみられる著しい FVIII : C の差異は、測定法に起因するものが大きいと推測される。今回の解析においても、患者 1 が最初に近医にて APTT 試薬としてアクチン FS (表 1 #2) を用いて測定した FVIII : C が約 60%であったのに対し、我々の研究室で HemosIL™ APTT-SP (表 1 #14) を用いて測定した結果が 34.5%であったことも、APTT 試薬を含めた測定法の違いが関与していると考えられる。しかしながら R531H 変異に限らず血友病 A では、von

Willebrand 因子量、血液型、ポリモルフィズム、日差などの要因が関与しているためか、同一の変異を有する患者であっても同一の FVIII : C やクリニカルフェノタイプとはならないことはしばしば経験されることであり、実際、今回検討した 2 例では同一の方法で測定された FVIII : C でありながら患者 1 の FVIII : C は約 1.7 倍高値であった。したがって、患者個人の要因のみならず、さらに検査法の要因が通常より大きく関与する R531H の FVIII : C 測定では、得られた値が本当に患者の病態を把握するために適正な値であるのかについては慎重に検討する必要がある。また、このような変異の場合、軽症血友病 A と診断されていないケースが多く存在するのではないかとすることも懸念される。通常、血友病 A の診断は、まず APTT によるスクリーニングを行い、延長が認められた症例に対して FVIII の定量が行われるが、患者 1 のようなラボラトリーフェノタイプでは、スクリーニングとして行われる APTT において明ら

かな延長が認められないため血友病Aの診断に至らない場合も少なくないと思像される。したがって、血友病AのスクリーニングおよびFVIIIの定量は、FVIIIに対する感受性の高いAPTT試薬を用いて行うことが重要であろうと考える。

活性化FVIII分子は、A1、A2およびA3-C1-C2の3つのサブユニットで構成されるヘテロトリマー構造をとっており、R531HはA2サブユニットに位置する。トロンビンによって活性化されたFVIIIのA2サブユニットは、他の2つのAサブユニットとの非共有結合にて複合体を形成しており、このA2サブユニットの解離はFVIIIの失活につながる¹⁰。Pipeら¹¹はリコンビナントFVIIIの実験から、R531Hでは野生型に比べA2サブユニットが解離しやすいことを確認しており、基質反応までの加温時間が長い2段階法では、A2サブユニットの解離の亢進によりFVIII:Cが低値となってしまうことを報告している。またRodgersら¹²は古典的な凝固2段階法と合成基質法の両法について加温時間の検討を行い、短い加温時間の方が高値を呈することを確認している。我々も凝固1段階法での加温時間について検討し、加温時間の延長による活性低下の度合いがR531Hでは正常FVIIIのそれに比べて顕著であり、R531Hが加温に対してより不安定であることを確認している(データ非提示)。これらのことから加温時間は乖離に関与する重要な要因であることは間違いない。しかし加温時間以外にも、試薬に起因する何らかの別の要因が乖離に関与することが今回の解析において確認され、各APTT試薬の構成成分とpHの検討を行った。APTT試薬の組成は製造メーカーが独自に培ってきた製造技術情報をもとに、自社の試薬に何らかの特徴を持たせるような工夫もなされており、その詳細が把握できないため、添付文章に記載されている限られた情報を参考に乖離に関連する因子の検討を行った。その結果、FVIII:Cと試薬構成成分であるリン脂質や活性化物質との間

には特定の関連を認めなかったが、pHとの間には相関関係が示唆された。Lollarら^{12,13}はブタのFVIIIの実験から、pHが高い条件ではA2サブユニットの解離が速やかになり、活性化FVIIIの凝固活性が速やかに減少することを確認している。またFayら¹⁴もヒトのFVIIIを用いた実験で同様の結果を報告している。今回得られたpHの影響は、これらの報告のpHの影響と一致しており、APTT試薬のpHがA2サブユニットの解離に影響しFVIII:Cを変動させる要因であると考えられた。一方、Perssonら¹⁵はA2サブユニット解離に関与するpH以外の要因として、イオン強度、カルシウムイオン濃度を報告しており、これらの影響も併存している可能性も考えられる。また今回は由来のみの調査であったが、リン脂質に関しては詳細な組成の検討により乖離との関与が認められる可能性がある。

APTT試薬のpHがFVIII:C測定、特にR531Hのような変異体のFVIII:C測定に重要であることが確認されたことから、反応液のpHを整えることは精密な検査を行ううえで有効であると考え、試薬と検体の希釈に3種類の緩衝液を用いその効果について検討と、緩衝液の効果は一様とはならず、イミダゾール緩衝液が最も効果的に作用した。イミダゾール緩衝液は凝固検査の際に用いられることが多く、FVIIIではインヒビターの測定法として一般的に知られているベセスダ法における検体の希釈液に用いられる。なぜイミダゾール緩衝液が最も有効に働いたかについては不明である。今後、#11と#14以外のAPTT試薬の場合や、他の緩衝液を用いた場合などの解析を行い、検討する必要がある。

今回検討したR531Hの2症例は共に臨床的には軽症型であるが、いずれの試薬を用いたFVIII:Cの結果が臨床症状を最も反映しているのかは不明である。同一変異を有する他の症例の詳細な臨床症状とFVIII:Cの関係を含めて検討をする必要がある。

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Features of factor VIII activity measurement in Arg531His mutant detected in patients with mild hemophilia A

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Key words: Mild hemophilia A, Mutation, One-stage clotting assay, Discrepancy

Arg531His (R531H) mutation was identified from unrelated two Japanese patients with cross reacting material positive (CRM+) mild hemophilia A. Although these patients have the same genetic defect and mild clinical phenotype, FVIII activity differed considerably, between the two. In Patient 1, FVIII activity, measured with 14 different APTT reagents, ranged widely, from 25.2% to 48.1%. There was a negative correlation between reagent pH and R531H measurement. Although the FVIII activity in Patient 2 also showed a discrepancy, the difference was considerably lessened by use of an imidazole buffer for sample dilution solution. This finding suggests that the use of imidazole buffer as a sample diluent is an effective means for reducing the wide range of assay results. As for the results of the present study, FVIII levels associated with the R531H mutation differed with the patients and were greatly influenced by measurement method. It is necessary to establish a standard method that can detect and characterize the mild hemophilia phenotype caused by a particular mutation, such as Arg531His.

Actual status of AIDS-related lymphoma management in Japan

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Recently, with the widespread use of highly active antiretroviral therapy (HAART), the occurrence of opportunistic infections as an acquired immunodeficiency syndrome (AIDS)-defining illness (ADI) has declined dramatically [1]. Decreases in the incidence of AIDS-related lymphoma (ARL) are not as evident compared with other ADI, so lymphoma has now become one of the most common ADIs [2]. Lymphoma has also become a more common cause of mortality due to AIDS. Management of ARL is starting to represent a critical problem in the total care of the Human Immunodeficiency virus (HIV)-infected population.

In Japan, no epidemiological survey of ARL has been systematically conducted, and the actual status of clinical practice for ARL has been unclear. We therefore made inquiries about ARL management to all hospitals with specialists in hematology (educational hospital certified by the Japanese Society of Hematology) and regional center hospital for HIV/AIDS (502 and 369 institutes, respectively; 209 overlapping institutes) in Japan. First, we asked about experience with ARL treatment and the department responsible for treating ARL in July 2007. Replies were obtained from 305 of the 502 hospitals with specialists in

hematology (72.9%) and 218 of the 369 regional center hospitals for HIV/AIDS (59.1%) [174 of 209 overlapping hospitals (83.3%)]. Experience with at least 1 ARL patient was reported by 143 of 349 institutes that responded to our inquiries (41.0%), and ARL was treated in the hematology department in about 80% of these institutes. Next, inquiries about the number of cases of ARL in the past 5 years and treatment modalities for ARL were made to these 143 ARL-experienced institutes. Of the 110 institutes that replied, 54 had treated ARL in the last 5 years (129 cases in total). Of these institutes, 43 treated ARL in the hematology department, and 8 in the infectious disease department (Table 1). These 8 infectious disease departments managed about 40% of all cases of ARL, and thus should be familiar with AIDS and ARL. However, 39 of the 43 hematology departments had experienced less than 2 patients in 5 years, and these institutes also had small numbers of AIDS patients (Table 2). In addition, 40% of ARL cases were treated in these 39 hospitals. The results of this survey suggest the presence of some gap in experience among hospitals treating ARL in Japan.

In the treatment of ARL, use of HAART is considered critical. Clinical studies have shown significant advantages in overall survival when HAART is combined with chemotherapy [3, 4]. However, our survey showed disagreement in the timing of starting HAART among institutes (Fig. 1). The majority of institutes administer chemotherapy against ARL currently with HAART, but 18% of institutes start HAART after completing chemotherapy.

Recently, very high efficacy of dose-adjusted EPOCH (etoposide, prednisone, vincristine, cyclophosphamide, and doxorubicin) for AIDS related aggressive B cell lymphoma was presented [5]. But, the standard chemotherapeutic regimen for ARL has not been established. This study

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Table 1 ARL-treating department

	Institutes	Patients
Hematology department	39	59
Infectious diseases department	8	52
Other	7	18
Total	54	129

The 54 institutes that replied to our questionnaires had treated 129 ARL patients between 2004 and 2007

Table 2 Differences in experience of ARL among institutes

Cases of ARL treated in 1 institute in the last 5 years	≥10	6–9	3–5	1–2
Number of institute treating ARL in hematology department	0	0	4	35
Number of institute treating ARL in infectious diseases department	2	1	2	3
Miscellaneous	0	0	3	4
Total	2	1	9	42

Number of institutes was indicated by the number of ARL cases treated between 2004 and 2007

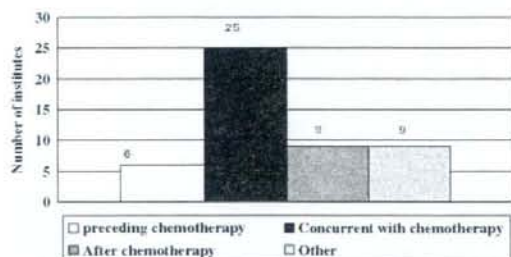


Fig. 1 Start of HAART in treatment of ARL with chemotherapy differs among institutes. A total of 49 institutes replied to this questionnaire

showed that chemotherapeutic regimens have not been standardized also in Japan. At the treatment for AIDS-related diffuse large B-cell lymphoma (DLBCL), the majorities of institutes have adopted EPOCH or CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) therapy. As indicated in this study, the combination of rituximab remains controversial for AIDS-related B-cell lymphoma (Fig. 2) [6, 7]. The addition of rituximab to chemotherapy improved the response rate, but increased the risk of infectious events. Rituximab should be used cautiously especially in low CD4 counts patients [8].

As shown earlier, management of ARL is inconsistent among institutes, and probably among individual doctors.

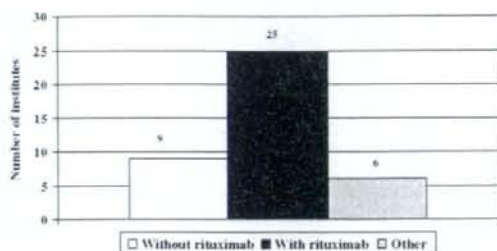


Fig. 2 Rituximab is not uniformly used for AIDS-related DLBCL (institutional policy). A total of 40 institutes replied to this questionnaire

Provision of appropriate guidelines for ARL treatment, including HAART, chemotherapeutic regimens and supportive care, thus seems important and urgent. A nationwide cooperative system of approaching ARL between hospitals, including epidemiological research, performance of clinical trials and consultation with patients needs to be established. Such steps would significantly contribute to the clinical care of ARL patients and might finally improve the quality of medication for the HIV-infected population in Japan.

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「血液凝固異常症のQOLに関する研究」

平成20年度調査報告書

「血液凝固異常症の QOL に関する研究 平成 20 年度調査報告書」の刊行に際して

この度、平成 20 年度厚生労働科学エイズ対策研究事業「血友病の治療とその合併症の克服に関する研究（研究代表者：坂田洋一）」の分担研究として行われた「血液凝固異常症の QOL に関する研究 平成 20 年度調査報告書」が出来上がりましたのでお送り申し上げます。

本報告書は昨年度の報告書に引き続き、自由記載を中心にした解析結果、3 年間の調査研究の総括、そして血液凝固異常症 QOL 調査運営委員からの行政、医療関係者、患者さんおよびご家族への提言を掲載しました。

本調査の実施に際しては、御多忙中にも拘わらず QOL 調査票に御記入頂き、返送して頂いた患者さんおよびその御家族の方に深謝申し上げます。また、本調査票の配布を仲介して頂いた患者組織および担当医の皆様にも厚く御礼申し上げます。本調査は、患者さんおよびご家族の治療および生活の質の向上に寄与できることを目的に行われました。今後、このような調査の機会がありましたら、今回指摘されました定年後（老後）の問題点や自立に関する質問含めた調査を行い、現状解析とその対策について提言をしてまいりたいと思います。

なお、昨年度、ホームページ（アドレスは <http://www.b-qol.com/>）に 19 年度報告書を掲載しましたが、20 年度報告書も近々掲載予定ですのでご覧ください。

平成 21 年 2 月

平成 20 年度厚生労働科学エイズ対策研究事業「血友病の治療とその合併症の克服に関する研究（研究代表者：坂田洋一）」分担研究：「血液凝固異常症の QOL に関する研究」

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I. 行政、医療関係者、患者さんおよびご家族への
血液凝固異常症 QOL 調査運営委員からの提言

1. 行政、医療関係者、患者さんおよびご家族への血液凝固異常症 QOL 調査運営委員からの提言

行政への提言

- 1) トータルケアの実践ができるよう積極的に地域別に血友病センターを創設する。
- 2) 安全な血液凝固因子製剤を安定供給する。
- 3) HIV 感染、HCV 感染に対する研究および治療を推進する。
- 4) 血液凝固異常症患者に対する公費負担制度を存続させ、併せて更新手続きを簡素化する。
- 5) 国民の血液凝固異常症患者への偏見・差別を払拭させる。
- 6) 疾患の治癒をめざした遺伝子異常を正常化させる治療法、遺伝子治療、細胞療法などの先進医療の研究を推進する。

医療関係者への提言

- 1) トータルケアの実践ができるように努める。
- 2) 血友病診療ネットワークの構築を行い、専門病院と地域の診療所、病院との役割分担をする。
- 3) 定期的にインヒビター、関節評価などを行い、適切な治療介入を行う。
- 4) 新規に診断された重症患者に対して関節障害発症予防や重篤な出血予防のため在宅自己注射に基づいた一次定期補充療法を患者・家族の負担にならぬよう工夫して導入する。
- 5) 既に関節障害があり、出血が多い患者に対して在宅自己注射に基づいた二次定期補充療法の導入と積極的な理学療法を行う。
- 6) 耐久性の優れた人工関節の開発、損傷した関節を治癒すべく再生医療の研究を行う。
- 7) インターフェロン未治療あるいはインターフェロン単独治療では完治しなかった HCV 感染者に対して、積極的にペグインターフェロンとリバビリンの併用療法を薦める。
- 8) 最新の医療情報を提供する。
- 9) 患者・家族会と連携する。
- 10) 遺伝子治療、細胞療法などの先進医療を研究し、臨床応用を図る。
- 11) 遺伝子異常を正常化させる治療法を研究し、疾患の治癒を図る。

患者さんおよびご家族への提言

- 1) 定期的に専門医療機関に受診し健康状態、特にインヒビター発生や関節状態の評価を受け、時期を逸することなく治療を受けましょう。
- 2) 在宅自己注射を早期に開始するよう努力しましょう。
- 3) 重症型の血友病と診断された保護者の方は専門医と相談の上積極的に一次定期補充療法の開始を考慮しましょう。
- 4) 関節障害があり、出血が多い場合は、二次定期補充療法の実施を考慮しましょう。
- 5) 自分自身（あるいはお子さん）の病気の理解を深める努力をしましょう。
- 6) 周囲の人たちへ病気に対する理解を求める努力をしましょう。
- 7) 主治医と相談の上できる範囲で学校行事やクラブ活動に積極的に参加しましょう。
- 8) 小児期から自己管理を試みるなど自立に向けた行動をしましょう。
- 9) 患者・家族会へ積極的に参加しましょう。
- 10) C 型肝炎にかかっている患者さんで、まだ一度もインターフェロン治療を受けたことがない場合や過去に行われたインターフェロン単独治療では完治しなかった場合には、ペグインターフェロンとリバビリンの併用療法を速やかに実施することをお勧めします。C 型肝炎は症状がなくとも病期は進行し、症状が出たときには肝硬変・肝癌になっている危険性が高いからです。主治医とよく相談して下さい。